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(71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 Cambridge Park Drive, Cambridge, MA 02140 (US).

(72) Inventors: SONG, Chuanzheng; 85 Brainerd Road, Apartment 207, Allston, MA 02134 (US). BROWN, Julie, C.; 46 Lebanon Street, Melrose, MA 02176 (US). LEEYING, Wu; 57 Brighton Avenue, Apartment 8, Allston, MA 02134 (US). RIVERA, Daniel, S.; 9 Thaxter Street, Hingham, MA 02043 (US).

(74) Agent: SPRUNGER, Suzanne, A.; American Home Products Corporation, Patent & Trademark Department – 2B, One Campus Drive, Parsippany, NJ 07054 (US).

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(54) Title: PRIMERS–ATTACHED VECTOR ELONGATION (PAVE): A 5'–DIRECTED CDNA CLONING STRATEGY

(57) Abstract

A novel method for preparing cDNA libraries is disclosed.

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PRIMERS-ATTACHED VECTOR ELONGATION (PAVE): A 5'-DIRECTED CDNA CLONING STRATEGY

FIELD OF THE INVENTION

The present invention provides a novel method for preparing cDNA libraries containing enhanced percentages of full-length cDNA inserts.

BACKGROUND OF THE INVENTION

Technology aimed at the production of cDNA libraries, which are important tools in the discovery of biologically relevant genetic sequences, often produces cDNA libraries that are far from perfect. cDNA libraries may contain a high percentage of molecules where the cDNA insert within the library vector is not full-length as compared to the naturally-occurring mRNA molecule from which the cDNA was derived. cDNA libraries, even those designed to be "directional" or having the cDNA insert present in a particular 5'->3' orientation relative to the vector sequences, often contain a high percentage of "flipped" inserts where the cDNA insert is oriented in the opposite orientation from that which is most desirable for characterization and expression of the cDNA insert. In addition, some cDNA libraries demonstrate a high incidence of multiple inserts, where unrelated cDNA molecules are aberrantly ligated into the same vector molecule.

There exists a need for novel methods of cDNA library production, and it is to such methods that the present invention is directed.

Construction of high quality cDNA libraries, with greater than 90% of the inserts being the full-length copy of the corresponding mRNA molecules, is crucial to the success of our effort to clone all the human genes encoding secreted proteins. Several factors contribute to the poor quality of cDNA libraries constructed using the conventional method, i. e., cDNA synthesis followed by ligation into plasmid or phage vectors. First, mRNA molecules may be degraded during RNA isolation and in the process of first strand cDNA synthesis. In addition, most mRNA samples are isolated from total cellular RNA using the oligo-dT capture protocol and, therefore, contaminated with partially-precessed poly(A) containing precursor RNA and partially degraded 3' portion of mRNA molecules. Second, during first-strand cDNA synthesis, reverse transcriptase tends to prematurely fall off the RNA templates due to RNA secondary structures or insufficient processivity of the enzyme itself. Third, the ligation step after ds cDNA synthesis may result in the following undesirable artifacts: A). Multiple cDNA inserts are ligated into the same vector due to the high insert/vector ratio used to increase the population of clones containing a cDNA insert. B). There is a high percentage (about 10%) of flipped cDNA insert when a unidirectional library is constructed. C) Contaminating DNA can be incorporated into the library. For example, some of the early libraries constructed by Clontech were contaminated by yeast chromosome DNA when yeast tRNA was used to precipitated the cDNA. Another example is that when the full-length cDNA was selected (Carninci, *et al.*, 1996), ligation of contaminating partial cDNA into the vector compromised the quality of library. D). There is a selection for smaller cDNA inserts since they are ligated more efficiently than larger ones.

Numerous efforts have been taken to increase the cloning efficiency from a definite amount of mRNA and/or to increase the proportion of the full-length inserts. Some of the most successful approaches include: A). An engineered reverse transcriptase was designed by GIBCO-BRL to inactivate its RNase H activity, which causes on-template RNA cleavage and premature termination of transcription when the enzyme stutters before a secondary structure. Thus far, the Superscript II reverse transcriptase (BRL) remains the most popular enzyme for first-strand

cDNA synthesis. B). Oligo-dT tailed vectors were used for first-strand cDNA synthesis (Okayama and Berg, 1982; Alexander et al., 1984; Bellemare et al., 1991; Kato et al., 1994). This method dramatically increased the cloning efficiency and the proportion of insert-containing clones. C). Strategies for specific capture (Edery et al., 1995) or labeling of the 5'-end cap of mRNA molecules with oligonucleotides (Fromont-Racine et al., 1993; Liu and Gorovsky, 1993; Maruyama and Sugano, 1994; Kato et al., 1994) or biotin (Carninci et al., 1996, 1997) were used to select for full-length cDNA. Libraries constructed with a selection for the 5'-end cap such as the Kato strategy (Kato et al., 1994, the Protagene protocol) and the biotin capture method (Carcinci et al., 1996) have a high percentage of full-length cDNA inserts ranging from 70% to 95%. However, none of the above mentioned strategies could completely satisfy the requirements for high efficiency, high proportion of full-length cDNA inserts and low contaminating or aberrant DNA inserts due to DNA ligation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the disclosed method for preparing mRNA molecules for cDNA library construction: mRNA is treated with phosphatase and then with pyrophosphatase, followed by ligation with RNA ligase to add an RNA tag to the 5' phosphate that will only be present on full-length mRNA molecules.

Figure 2 is an autoradiograph of a Northern blot showing the ligation of tobacco acid pyrophosphatase (TAP)-treated (lanes 1 and 2) or capped (no TAP treatment, lane 3) rabbit globin mRNA with either an RNA tag (lanes 1 and 3) or a DNA tag (lane 2) using T4 RNA ligase. The blot was hybridized with an radioactively labeled oligodeoxynucleotide complementary to the tag sequence. The arrow points to the position of full-length tagged rabbit globin mRNA. This Northern blot indicates that TAP treatment is necessary for efficient RNA ligation, and that, as compared to DNA tags, RNA tags are more efficiently ligated to mRNA molecules by T4 RNA ligase.

Figure 3 is a schematic representation of the pED6pdc4 vector that may be used for construction of cDNA libraries as disclosed herein, and includes the nucleotide sequence of the polylinker region of the pED6pdc4 vector.

Figure 4 is a schematic representation of the pED6pdc2 vector from which the pED6pdc4 vector was derived, and includes the nucleotide sequence of the polylinker region of the pED6pdc2 vector.

Figure 5 is another schematic representation of the pED6pdc2 vector and contains more information concerning the attributes of the pED6pdc2 vector. The pED6dpc2 vector was derived from pED6dpc1 by insertion of a new polylinker to facilitate cDNA cloning (Kaufman *et al.*, 1991, *Nucleic Acids Res.* 19: 4485-4490).

Figure 6 is a nucleotide sequence alignment that shows in detail the nucleotide differences between the pED6pdc2 and pED6pdc4 vectors.

Figure 7 is a schematic representation of the pED6pdc4 vector that may be used for construction of cDNA libraries as disclosed herein, and shows that the vector is digested with certain restriction enzymes and ligated to particular 5' and 3' linkers to form a pED6pdc4 vector-primer construct.

Figure 8 is a schematic representation of the pAVE1 vector that may be used for construction of cDNA libraries as disclosed herein, and shows that the vector is digested with certain restriction enzymes and ligated to particular 5' and 3' linkers to form a pAVE1 vector-primer construct.

Figure 9 is a schematic representation of the pNOTs vector from which the pAVE1 vector was derived. The pNOTs vector was derived from pMT2 (Kaufman *et al.*, 1989, *Mol. Cell. Biol.* 9: 946-958) by deletion of the DHFR sequences, insertion of a new polylinker, and insertion of the M13 origin of replication in the ClaI site.

Figure 10 is a schematic representation showing the creation of cDNA libraries by the combination of RNA-tagged mRNA molecules and pED6pdc4 vector-primer construct molecules, followed by first-strand synthesis (annealing and elongation by reverse transcriptase), RNAse digestion, intramolecular renaturation, and second-strand synthesis.

Figure 11 is a schematic representation showing the creation of cDNA libraries by the combination of RNA-tagged mRNA molecules and pAVE1 vector-primer construct molecules, followed by first-strand synthesis (annealing and elongation by reverse transcriptase), RNAse digestion, intramolecular renaturation, and second-strand synthesis. Note that in this figure the sequence at the 3′ end of the Vector-Primer construct has been reversed: the 3' should be shown as $NV(T)_{48}$ as in the 3′ linker shown in Figure 8.

Figure 12 is an agarose gel of digested cDNA clones showing the results of using the Primers-Attached Vector Elongation (PAVE) strategy with RNA-tagged globin mRNA: approximately 80% of the globin cDNAs are the expected size for full-length cDNA inserts (arrow), while for the untagged RNA controls full-length cDNA inserts are present at a much lower frequency.

Figure 13 shows schematically the structure of an RNA-tagged CPLA2-γ mRNA molecule used in the experiments of Figures 13-17.

Figure 14 shows schematically the structures and predicted sizes (as number of nucleotide residues) of different probe-RNA hybrids that could result from RNA-RNA ligation followed by RNAse digestion to remove single-stranded RNA.

Figure 15 is a digitized scan of radioactively detected RNA molecules separated electrophoretically on a gel, showing the effect of ATP concentration upon the efficiency of the reaction adding a RNA tag to a mRNA molecule using T4 RNA ligase. Arrows show the expected sizes for ligated and unligated molecules. At a relative concentration of 0.1X (5.8 nM ATP), 50.8 percent of the radioactivity detected was present as ligated molecules as compared to unligated molecules.

Figure 16 is a digitized scan of cDNA molecules separated electrophoretically on an agarose gel, showing that T7 polymerase is the most effective in completion of second-

strand synthesis as compared to T4, PFU (Promaga, Madison WI), and SEQUENASE (Amersham Pharmacia Biotech) DNA polymerases.

Figure 17 is a digitized scan of cDNA molecules separated electrophoretically on a series of agarose gels, showing that the inclusion of tRNA in the RNAse digestion reaction prior to the second-strand synthesis reaction does not result in the inclusion of tRNA molecules in the cDNA reaction products. Further, this Figure shows that cDNA molecules produced without a second-strand synsthesis ("Annealed" in the Figure) are capable of being transformed into host cells and are maintained therein.

DETAILED DESCRIPTION

The following examples, tables, and figures provide examples of ways in which the methods of the present invention may be accomplished. These examples are not intended to limit in any manner the number of ways in which these methods may be carried out by those of skill in the art, or the types of vectors, primers, and other materials that may be utilized in these methods. In particular, those of skill in the art will appreciate that by selecting different sequences for the 5' and 3' linkers (also interchangeably called primers throughout) of the present method, linkers (or primers) can be designed that will anneal to any vector of known nucleotide sequence digested with any particular restriction enzyme(s).

For example, the invention also includes polynucleotides with sequences complementary to those of the polynucleotides disclosed herein. The present invention also includes polynucleotides which are derived from the polynucleotides disclosed herein by any of the following or by a combination thereof: addition of residues; deletion of residues; substitution of residues, whether with polynucleotide residues or other molecules such as amino acids, carbohydrates, lipids, or modified forms thereof; or chemical modification of existing residues. Examples of chemical modifications include but are not limited to methylation, addition of other alkyl groups, addition of aromatic or heterocyclic molecules, addition or removal of a hydroxyl group, addition of polyethylene glycol, addition of carbohydrate, polypeptide, or lipid molecules, etc.

The present invention also includes polynucleotides that hybridize under reduced stringency conditions, more preferably stringent conditions, and most preferably highly stringent conditions, to polynucleotides described herein. Examples of stringency conditions are shown in the table below: highly stringent conditions are those that are at

least as stringent as, for example, conditions A-F; stringent conditions are at least as stringent as, for example, conditions G-L; and reduced stringency conditions are at least as stringent as, for example, conditions M-R.

Stringency Condition	Polynucleotide Hybrid	Hybrid Length (bp)‡	Hybridization Temperature and Buffer†	Wash Temperature and Buffer†
A	DNA:DNA	≥ 50	65°C; 1xSSC -or- 42°C; 1xSSC, 50% formamide	65°C; 0.3xSSC
B	DNA:DNA	<50	T_B*; 1xSSC	T_B*; 1xSSC
C	DNA:RNA	≥ 50	67°C; 1xSSC -or- 45°C; 1xSSC, 50% formamide	67°C; 0.3xSSC
D	DNA:RNA	<50	T_D*; 1xSSC	T_D*; 1xSSC
E	RNA:RNA	≥ 50	70°C; 1xSSC -or- 50°C; 1xSSC, 50% formamide	70°C; 0.3xSSC
F	RNA:RNA	<50	T_F*; 1xSSC	T_F*; 1xSSC
G	DNA:DNA	≥ 50	65°C; 4xSSC -or- 42°C; 4xSSC, 50% formamide	65°C; 1xSSC
H	DNA:DNA	<50	T_H*; 4xSSC	T_H*; 4xSSC
I	DNA:RNA	≥ 50	67°C; 4xSSC -or- 45°C; 4xSSC, 50% formamide	67°C; 1xSSC
J	DNA:RNA	<50	T_J*; 4xSSC	T_J*; 4xSSC
K	RNA:RNA	≥ 50	70°C; 4xSSC -or- 50°C; 4xSSC, 50% formamide	67°C; 1xSSC
L	RNA:RNA	<50	T_L*; 2xSSC	T_L*; 2xSSC
M	DNA:DNA	≥ 50	50°C; 4xSSC -or- 40°C; 6xSSC, 50% formamide	50°C; 2xSSC
N	DNA:DNA	<50	T_N*; 6xSSC	T_N*; 6xSSC
O	DNA:RNA	≥ 50	55°C; 4xSSC -or- 42°C; 6xSSC, 50% formamide	55°C; 2xSSC
P	DNA:RNA	<50	T_P*; 6xSSC	T_P*; 6xSSC
Q	RNA:RNA	≥ 50	60°C; 4xSSC -or- 45°C; 6xSSC, 50% formamide	60°C; 2xSSC
R	RNA:RNA	<50	T_R*; 4xSSC	T_R*; 4xSSC

‡: The hybrid length is that anticipated for the hybridized region(s) of the hybridizing polynucleotides. When hybridizing a polynucleotide to a target polynucleotide of unknown sequence, the hybrid length is assumed to be that of the hybridizing polynucleotide. When polynucleotides of known sequence are hybridized, the hybrid length can be determined by aligning the sequences of the polynucleotides and identifying the region or regions of optimal sequence complementarity.

†: SSPE (1xSSPE is 0.15M NaCl, 10mM NaH_2PO_4, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes after hybridization is complete.

*T_B - T_R: The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, T_m(°C) = 2(# of A + T bases) + 4(# of G + C bases). For hybrids between 18 and 49 base pairs in length, T_m(°C) = 81.5 + 16.6($\log_{10}$[Na^+]) + 0.41(%G+C) - (600/N), where N is the number of bases in the hybrid, and [Na^+] is the concentration of sodium ions in the hybridization buffer ([Na^+] for 1xSSC = 0.165 M).

Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook, J., E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, chapters 9 and 11, and *Current Protocols in Molecular Biology*, 1995, F.M. Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4, incorporated herein by reference.

Preferably, each such hybridizing polynucleotide has a length that is at least 25%(more preferably at least 50%, and most preferably at least 75%) of the length of the polynucleotide of the present invention to which it hybridizes, and has at least 60% sequence identity (more preferably, at least 75% identity; most preferably at least 90% or 95% identity) with the polynucleotide of the present invention to which it hybridizes, where sequence identity is determined by comparing the sequences of the hybridizing polynucleotides when aligned so as to maximize overlap and identity while minimizing sequence gaps.

In particular, sequence identity may be determined using WU-BLAST (Washington University BLAST) version 2.0 software, which builds upon WU-BLAST version 1.4, which in turn is based on the public domain NCBI-BLAST version 1.4 (Altschul and Gish, 1996, Local alignment statistics, Doolittle *ed.*, *Methods in Enzymology* **266**: 460-480; Altschul *et al.*, 1990, Basic local alignment search tool, *Journal of Molecular Biology* **215**: 403-410; Gish and States, 1993, Identification of protein coding regions by database similarity search, *Nature Genetics* 3: 266-272; Karlin and Altschul, 1993, Applications and statistics for multiple high-scoring segments in molecular sequences, *Proc. Natl. Acad. Sci. USA* **90**: 5873-5877; all of which are incorporated by reference herein). WU-BLAST version 2.0 executable programs for several UNIX platforms can be downloaded from ftp://blast.wustl.edu/blast/executables. The complete suite of search programs (BLASTP, BLASTN, BLASTX, TBLASTN, and TBLASTX) is provided at that site, in addition to several support programs. WU-BLAST 2.0 is copyrighted and may not be sold or redistributed in any form or manner without the express written consent of the author; but the posted executables may otherwise be freely

used for commercial, nonprofit, or academic purposes. In all search programs in the suite -- BLASTP, BLASTN, BLASTX, TBLASTN and TBLASTX -- the gapped alignment routines are integral to the database search itself, and thus yield much better sensitivity and selectivity while producing the more easily interpreted output. Gapping can optionally be turned off in all of these programs, if desired. The default penalty (Q) for a gap of length one is Q=9 for proteins and BLASTP, and Q=10 for BLASTN, but may be changed to any integer value including zero, one through eight, nine, ten, eleven, twelve through twenty, twenty-one through fifty, fifty-one through one hundred, etc. The default per-residue penalty for extending a gap (R) is R=2 for proteins and BLASTP, and R=10 for BLASTN, but may be changed to any integer value including zero, one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve through twenty, twenty-one through fifty, fifty-one through one hundred, etc. Any combination of values for Q and R can be used in order to align sequences so as to maximize overlap and identity while minimizing sequence gaps. The default amino acid comparison matrix is BLOSUM62, but other amino acid comparison matrices such as PAM can be utilized.

A number of types of cells may act as suitable host cells to be transformed with the products of the cDNA library preparation reactions. Mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells. Alternatively, it may be possible to use host cells such as lower eukaryotes like yeast or prokaryotes such as bacteria. Potentially suitable yeast strains include *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces* strains, *Candida,* or any yeast strain capable of being transformed with cDNA clones. Potentially suitable bacterial strains include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of being transformed with cDNA clones.

Patent and literature references cited herein are incorporated by reference as if fully set forth.

In this proposal, we describe an improved strategy (compared to Kato et al., 1994) that we call Primers-Attached-Vector Elongation (PAVE). The crucial element of the strategy is a novel vector attached with primers for both first strand and second strand cDNA synthesis . The oligo-dT primer attached to one end of the vector is used to prime first-strand cDNA synthesis from the poly(A) stretch of the mRNA, whose cap has been specifically labeled with a 27-mer biotinylated RNA tag. After digestion of the single-stranded RNA with RNase I, full-length cDNA is captured by streptavidin beads. Second strand synthesis is then carried out using the primer (with sequence identical to the RNA tag) at the other end of the vector, which would specifically base pair with a full-length cDNA that contains a sequence complementary to the RNA tag. This will give rise to a circularized plasmid for subsequent E. coli transformation. Since no DNA ligation will be necessary after cDNA synthesis, all the possible artifacts generated by cDNA-vector ligation will be theoretically eliminated. In addition, the availability of double-strand vectors containing single-strand cDNA inserts before the second strand cDNA synthesis provides a mechanism for library normalization and subtraction and would also allow subgrouping the cDNA libraries into the subset encoding secreted and membrane proteins and the subset encoding soluble proteins.

Examples

Example 1
Preparation of Vector-Primer

Plasmid vector pED6dpc4 was completely digested with EcoR I and Sal I. Thirty micrograms of digested plasmid DNA was then ligated with 840 pmol each of the following two linkers:

Linker 1

Phosphate-5'-AATTCGAGTGAACACTCGAGCTCACTAGTGACCAGCTGATGCGCCTCAAA-3' (SEQ. ID. #1)
3'-GCTCACTTGTGAGCTCGAG-5' (SEQ. ID. #2)

Linker 2

5'-CTAATCTGATCCGCTAGTGGTAC-3' (SEQ. ID. #3)
3'-$(T)_{30}$GATTAGACTAGGCGATCACCATGAGCT-5'-Phosphate (SEQ. ID. #4)

in a 1.4 ml reaction volume using T4 DNA ligase (NEB) under conditions suggested by the manufacturer. The ligated plasmid DNA was then purified through electrophoresis on a 0.8% agarose gel.

Example 2
Ligation of a Biotinylated RNA Tag to the 5'-end of Full-length mRNA

Ten ug of rabbit globin mRNA was treated with 5 units of HK phosphatase (Epicentre) in a total volume of 250 ul under conditions recommended by the manufacturer. After incubation at 37 oC for 30 min, the mixture was extracted with phenol/chloroform and precipitated with NaOAc/ethanol. The pellet was dissolved in 20 ul of DEPC-treated water and 19.5 ul of which was subjected to digestion with 5 units of tobacco acid pyrophosphatase (TAP) in a 50 ul volume. The reaction was carried out at 37 oC for 30 min and terminated by phenol/chloroform extraction. After NaOAc/ethanol precipitation, the pellet was dissolved in 20 ul of DEPC-treated water. Fifteen ug of TAP treated RNA was then ligated to 7 ug of RNA tag (27-mer synthetic ribonucleotide with 5' biotin group) in a 120 ul reaction mixture containing 50 mM Tris-Cl, pH 7.8, 10 mM MgCl2, 10 mM DTT, 1 mM ATP and 12 units of T4 RNA ligase (Takara). After overnight incubation at room temperature, the sample was extracted twice with phenol/chloroform and precipitated with NaOAc/ethanol. The

pellet was dissolved in DEPC-treated water.

As a control experiment, 2.5 ug of the TAP treated RNA was ligated to 2.5 ug of 5' biotinylated DNA tag in a reaction volume of 40 ul and the sample was treated as described above.

To assess the efficiency for ligating the RNA or DNA tag to rabbit globin mRNA, 0.25 ug of the RNA samples were electrophoresized on a 4-20% TBE/PAGE minigel (Novex) and blotted onto nylon-plus membrane (QIAGEN). After hybridization with 32P-labeled anti-tag (SEQ ID # 5'-GAGGCGTATCAGCTGGTCACT-3') according to Sambrook et al., 1989, the position of mRNA molecules ligated with either the RNA or DNA tag was revealed by autoradiography. As judged from Figure 4, RNA tag is ligated to the TAP-treated mRNA much more efficiently than the DNA tag.

Example 3
cDNA Synthesis and Cloning

Approximately 1.25 ug of biotin-RNA-tagged mRNA was mixed with 1.2 ug of vector-primer in a final volume of 20 ul containing 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM each of the four dNTPs and 200 units of Superscript II (GIBCO BRL) and the reaction was carried out at 48 oC for 1 hour. The cDNA was then extracted with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in water and digested with 25 units of RNase One (Promega) and 6 units of E. Coli RNase H (Epicentre) in 60 ul of reaction mixture containing 10 mM Tris-Cl, pH 7.9, 10 mM MgCl2, 50 mM NaCl and 1 mM DTT. After 1 hour incubation at 37 oC, 30 ul of water and 10 ul of 10 X annealing buffer (0.5 M Tris-Cl, pH 8.0, 0.1 M MgCl2 and 0.5 M NaCl) were added and the mixture was heated at 70 oC for 5 min and slowly cooled down to 50 oC in 30 min. Ten ug of glycogen was then added the DNA was precipitated in NaOAc/ethanol.

For second-strand cDNA synthesis, the above DNA pellet was dissolved in 13 ul of water and 2 ul of 10 X T4 DNA polymerase buffer (NEB), 4 ul of dNTPs (2.5 mM each), 1 ul of 1 mg/ml of BSA and 1 ul (3 units) of T4 DNA polymerase were subsequently added. After 1 hr at 37 oC, the DNA was precipitated and used to transform competent E. coli cells (DH10B, GIBCO BRL).

When tagged rabbit globin mRNA was used in the above procedure, the efficiency of the library is about 10^6 colonies/ug of starting mRNA.

When plasmids were isolated from randomly picked individual colonies and digested withAsc I and Not I to release the insert, 37 out of 48 clones have full-length (about 650 bp) cDNA inserts. In addition, 5'-end and 3'-end DNA probes were used to hybridize to duplicate filters lifted from plated colonies and 75.8% of the colonies are full-length as judged by being able to hybridize to both probes (Table 1).

Experimental Design And Expected Results

I. Construction of a multi-purpose vector (pAVE1) for in vitro and in vivo protein expression

A vector pAVE1 has been constructed for our large scale molecular biology effort to obtain the full-length cDNAs of all the human secreted proteins in a single cloning step. pAVE1 is derived from pNOTS by replacing its Pst I/Xho I fragment with a 100 bp designed linker. Some of the notable features of pAVE1 include:

A). T7 and T3 RNA polymerase promoters flanking the cDNA insert to be cloned from 5' to 3' into the Eco RI and Kpn I sites, allowing sense and anti-sense RNA molecules to be synthesized, respectively. The T7 RNA promoter also allows coupled in vitro transcription and translation (TNT) protocol to be used to assess the size of the encoded protein products.

B). Four eight-base recognizing restriction sites flanking T7 and T3 promoters, permitting easy subcloning of the cDNA inserts.

C). Suitable for COS expression because of the SV40 origin and the eukaryotic expression cassette.

D). The f1 origin (from the pNOTS backbone) would allow ssDNA to be prepared for library subtraction and normalization. In addition, recombinant f1 phage particles can be used to transfect COS cells (Yokoyama-Kobayashi and Kato, 1993). If we could engineer a patentable COS cell line that can specifically and efficiently endocytosize f1 phage particles, then we can carry out COS transfection in a large scale fashion without the need for plasmid preparation.

II. Preparation of primers-attached-vector

Eco RI and Kpn I digested pAVE1 plasmid DNA will be gel-purified and ligated to the 5'-end linker, which is compatible with the Eco RI end and contains a single-stranded sequence identical to the RNA tag, and to the 3'-end linker, which is compatible with the Kpn I end and contains single-strand oligo-dT sequence. The ligated DNA product will be gel-purified

and the presence of the primers will be confirmed by digestion with Hind III and Bst XI followed by polyacrylamide gel analysis More than 90% of the vector should be attached with the two primers if the proper linker/vector ratio is used. Otherwise, the desired primers-attached vector DNA should be purified by consecutive oligo-dA column and anti-RNA tag oligonucleotide column.

III. Tagging the cap of the mRNA with oligoribonucleotides

The mRNA samples will be treated with the heat-killable (HK) phosphatase isolated from an antarctic bacterium (Epicenter) to remove the phosphate group at the 5' ends of degraded RNA molecules The cap of the full-length RNA population will be removed with tobacco acid pyrophosphatase (TAP; Shinshi et al., 1976a and 1976b; Efstratiadis et al., 1977; Fromont-Racine, et al., 1993; Maruyama and Sugano, 1994; Kato et al., 1994). The decapped mRNA molecules will then be ligated to a 27-mer biotinylated oligoribonucleotide (RNATAG, using T4 RNA ligase. The small RNA tag was the removed by repetitive ethanol precipitation.

There are two limitations for this procedure, i. e. the low ligation efficiency (about 60%, Tessier, et al., 1986) and the small proportion of mRNA-mRNA ligation. However, since selection of full-length cDNA will be applied after first strand cDNA synthesis (RNase I digestion followed by streptavidin capture) and during second strand synthesis (specific priming from the vector-attached primer), this may not have a great detrimental effect on the quality of the cDNA library (although it can reduce the number of colonies produced from a definite amount of mRNA).

IV. First strand cDNA synthesis and full-length cDNA enrichment

The tagged mRNA will be annealed to the primers-attached-pAVE1 vector and first strand cDNA synthesis will be carried out using Superscript II reverse transcriptase (GIBCO-BRL,). The first strand cDNA, together with the associated mRNA template, will be precipitated and subject to RNase I digestion to degrade unprotected single-strand RNA regions as well as unreacted free mRNA molecules In this reaction, only the biotin group of the mRNA whose cDNA

is full-length will be protected from clipping off the vector-primer-cDNA assembly. The full-length cDNA-vector molecules will then be captured using streptavidin magnetic beads and subject to complete RNase H and alkaline hydrolysis to remove the RNA strand. This will produce a population of single-strand full-length cDNA covalently linked to the pAVE1 vector through the poly (A/T) region. The full-length cDNA population will account for about 7-10% of the total cDNA synthesized by reverse transcriptase according to Carninci et al., 1996.

V. Second strand cDNA synthesis and transformation

The cDNA-vector molecules will be diluted, denatured and reannealed to allow base pairing between the vector-attached primer and the extreme 3' end of the single-strand full-length cDNA . Second strand cDNA will be synthesized using T4 DNA polymerase. The resulting double-stranded circular DNA (with two gaps at each end of the cDNA) will be used to transform E. coli strain 10B or DH5α. More than 10^6 primary colonies should be obtained for each microgram of vector-primer.

VI. Assessment of the quality of the cDNA library

A). Globin mRNA control

Pure globin mRNA (about 700 bases for both subunits) will be used to prepare a PAVE cDNA library. Duplicate filters from plates containing a total number of at least 10, 000 colonies will be hybridized with the 5'-end probe and the 3'-end probe, respectively. The ratio of 5'-end positive clones to the 3'-positive clones should be close to 1. At least 100 primary colonies will be picked for plasmid DNA preparation. Insert size will be determined by Asc I/Not I digestion. At least 90% of the colonies should have a full-length cDNA insert.

B). A real cDNA library

A PAVE cDNA library will be made from some mRNA isolated from a human tissue source, preferably pancreas. The GAPDH 5'- and 3'-end probes will be used for colony hybridization to assess the ratio of clones containing GAPDH cDNA inserts with 5' and 3' sequences. If the ratio is

close to 1 as expected, 300 colonies will be randomly picked from the entire library for plasmid preparation and the insert size will be determined for each clone. More than 95% of the clones should have a cDNA insert. In addition, the plasmid DNA sample will be subject to coupled in vitro transcription and translation (TNT) analysis in the presence of ^{35}S-labeled methionine. The size of the synthesized protein will be analyzed by 4-20% SDS-PAGE followed by autoradiography. If more than 90% of the insert-containing clones give rise to a protein product in the TNT assay, 3000 colonies will be subjected to 5'-end sequencing and the data will be subjected to bioinformatics evaluation.

An additional, and perhaps more rigorous, approach to evaluate quality of the library is to screen for the presence of the 7 kb full-length cDNA for human cPLA2β, whose mRNA is ubiquitously expressed but most abundant in pancreas. Previous effort) has produced more than 100 positive clones from four cDNA libraries and none of them is full-length (Song, Kriz, Bean and Knopf, unpublished).

Future Considerations

the following efforts should be considered to expedite our progress in cloning all the human cDNAs for secreted or membrane proteins and to facilitate their functional analysis:

I. Enrichment of cDNAs for secreted and membrane proteins

Strategy 1: Highly pure rough ER will be isolated by refining the sucrose-density centrifugation parameters. The mRNA molecules will be isolated, their poly A tails removed by oligo (dT)-directed RNase H digestion and the 5'-end cap labeled by biotin (Carninci, et al., 1996). The labeled rough ER mRNA will be hybridized with the single-stranded cDNA-vector population prepared from high quality total mRNA. After capture with streptavidin beads, the bound cDNA will be eluted and used to prepare a subset of cDNA library which should be highly enriched in cDNA molecules for secreted or membrane-bound proteins.

Strategy 2: Explore the possibility of in vitro TNT based library

subgrouping: Plasmid DNA from a PAVE cDNA library will be prepared and subject to in vitro TNT for a defined length of time. Inhibitors for T7 RNA polymerase and the translation machinery will be added to freeze the cDNA-RNA-nascent peptide complex. If the nascent peptide contains a secretion signal, the complex will be captured by a solid phase conjugated with signal recognition particle (SRP). The captured cDNA-vector population will be used to transform E. coli cells to create a subset enriched in cDNAs for proteins with a signal peptide.

II. Subtraction

The full-length cDNA clones for the most abundant mRNA species will be obtained when we sequence our first 3000 clones for library quality assessment. These clones will be collected and biotinylated sense RNA transcripts will be made from the Not I linearized plasmid DNA using T7 RNA polymerase. After removal of the 5' and 3' vector sequences on the RNA using an oligonucleotide-directed RNase H digestion approach, the remaining RNA will be used to subtract their corresponding cDNAs from the single-strand cDNA-vector population. The remaining cDNA-vector population should be enriched with rare messages.

III. Normalization

Normalization of PAVE libraries could be carried out before the initial bacteria transformation step, unlike in the original normalization protocol where amplified single-strand phagmid DNA was used (Soares, et al., 1994). Therefore, normalized PAVE cDNA libraries should have the same cDNA representation as the unnormalized primary library, minimizing the chance of losing some cDNAs that are selected against during amplification.

IV. An ES cell line library?

If we succeed in constructing normalized PAVE cDNA library with more than 95% of the inserts being full-length and encoding a protein product by TNT assay, then we can design a special vector which can direct the recombination of the cDNA insert into a specific locus in the mouse genome. Linearized plasmid DNA prepared from the library will be

used to transfect ES cells. The ES clones containing individual cDNA inserts at the expected location will be isolated and the identity of the cDNA analyzed by PCR and sequencing. Eventually, we should be able to establish an ES cell line library for convenient transgenic mice production. This is opposite to the Merck-Lexicon approach, where ES cell lines with disrupted genes are collected for production of knock-out mice, but maybe more relevant to the drug-discovery scenario, since most drugs are inhibitors to a disease target.

Tagging of mRNA

*****Do all RNA set-up in tissue culture hood********
** Do the following in silconized RNASE-FREE 1.5 ml tubes (Ambion).
ALL reagents are made in DEPC-WATER (Ambion).
Use only ART tips for all reactions.
Clean pipettes with RNASE AWAY and EtOH.
Place a new piece of lab paper on your bench (plastic side up).
Wear gloves at all times!!!!
IN GENERAL, CLEAN UP YOUR WORK AREA!!!!!!!
(RNASES are EVERYWHERE.)

DAY ONE:

Today: We are using 0.24-9.5KB markers (1µg/µl), TF-1 mRNA (1µg/µl) & Globin mRNA (1 µg/µl)
Turn the heating block on to 37 °C.

1 µl	tRNA (5µg/µl)		
36µl/39 µl	DEPC-water		(Ambion)
5 µl	10X BAP Buffer		(Gibco)
0.75 µl	0.1 M DTT		(Homemade-Sigma)
1.25 µl	RNAsin	(40 u/µl)	(Promega)
5µl/2 µl	mRNA	(1µg/µl)	(2 µg)
1 µl	BAP	(150 u/µl)	(Gibco)

V_T = 50 µl

* Incubate at 37 °C for 0.5 hour on a heating block with cover (pipette box top). If there is condensation, then do a quick spin.
* Add 100µl of DEPC-water then add 150 µl of phenol/$CHCl_3$/IAA pH 7.9 (Ambion) and "flick" for 0.5 min. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place into new 1.5 ml RNASE-FREE tube.
* Add 125 µl of DEPC-water (Ambion) to the original tube (bottom)) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place with the other aqueous layer in the 1.5 ml RNASE-FREE tube.
* Add 25 µl 3M NaOac, pH 4.5 (Autoclaved from media prep) and 625 µl of 100% EtOH. Incubate on dry ice for 5-8 minutes.
* Spin for 10-15 minutes at 4 °C at 14,000 rpm. Remove and SAVE (in a 1.5 ml RNASE-FREE tube) all of the EtOH layer except approximately 50 µl. Spin as above for 5 minutes. Remove the remaining EtOH without disrupting the pellet. Wash pellet with 200 µl of 80% EtOH chilled at –20 °C and spin for 2-5 minutes at 4 °C at 14,000 rpm. Remove EtOH and again spin for 1 minute at 14,000 RPM

and remove the remaining 1-5 µl of EtOH by just touching a 20 µl pipette tip to the edge of the drop of EtOH. Air dry with lids open on ice for 5 minutes.

- Resuspend in 20 µl DEPC-Water (Ambion) (100 ng/µl)
  ***** Save 500 ng (5 µl) of RNA markers **only**.....

1 µl	tRNA (5µg/µl)	
21.7 µl/26.7 µl	DEPC-water	(Ambion)
5 µl	10X TAP buffer	(Epicenter)
1.3 µl	RNAsin	(Promega)
20 µl/ 15µl	"BAP-ed" mRNA	
1 µl	TAP (10u/µl)	(Epicenter)
Vt= 50 µl		

* Incubate at 37 °C for 0.5 hour on a heating block with cover (pipette box top). If there is condensation, then do a quick spin.
* Add 150 µl water. Add 150 µl of phenol/$CHCl_3$/IAA pH 7.9 (Ambion) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place into new 1.5 ml RNASE-FREE tube.
* Add 125 µl of DEPC-water (Ambion) to the original tube (bottom)) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place with the other aqueous layer in the 1.5 ml RNASE-FREE tube.
* Add 25 µl 3M NaOAc, pH 4.5 (Autoclaved from media prep) and 625 µl of 100% EtOH. Incubate on dry ice for 5-8 minutes.
* Spin for 10-15 minutes at 4 °C at 14,000 rpm. Remove and SAVE (in a 1.5 ml RNASE-FREE tube) all of the EtOH layer except approximately 50 µl. Spin as above for 5 minutes. Remove the remaining EtOH without disrupting the pellet. Wash pellet with 400 µl of 80% EtOH chilled and spin for 2-5 minutes at 4 °C at 14,000 rpm. Remove EtOH and again spin for 1 minute at 14,000 RPM and remove the remaining 1-5 µl of EtOH by just touching a 20 µl pipette tip to the edge of the drop of EtOH. Air dry with lids open on ice for 5 minutes.
* Resuspend in 20 µl DEPC-Water (Ambion) (75 ng/µl)
* Save 500 ng (6.7 µl) of RNA markers **only**.....

** Ligase Buffer: 0.25 M Tris pH7, 0.25 M Tris pH8, 0.1M $MgCl_2$ (ALL Ambion Solutions)

** You have approximately 2 µg to ligate at this point.

** (1) RNA Markers, (2) Globin, (3) TF-1 mRNA

1µl	tRNA	(5µg/µl)	
56.95µl58 µl/ 64.7 µl	DEPC-water		(Ambion)
10 µl	10X NEW Ligase Buffer		(HOMEMADE--see recipe)
1 µl	1M DTT		(HOMEMADE--see recipe)
2.5 µl	RNAsin	(40 u/µl)	(Promega)
1.8 µl	FRESH 10 mM ATP		(Gibco-BRL)
1.75µl/0.7 µl/0.7µl	RNA-TAG	(100 pmol/µl)	(IDT)
20µ/20 µl/ 13.3 µl	TAP-treated mRNA	(2 µg)	(ABOVE reaction)
5 µl	T4 RNA Ligase	(5u/µl)	(GIBCO-BRL)

V_T = 100 µl

* Incubate at 16°C for 16 hours (overnight).

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* Add 50 µl of DEPC-water. Add 150 µl of phenol/$CHCl_3$/IAA pH 7.9 (Ambion) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place into new 1.5 ml RNASE-FREE tube.
* Add 125 µl of DEPC-water (Ambion) to the original tube (bottom)) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place with the other aqueous layer in the 1.5 ml RNASE-FREE tube.
* Add 25 µl 3M NaOAc, pH 4.5 (Autoclaved from media prep) and 625 µl of 100% EtOH. Incubate on dry ice for 5-8 minutes.
* Spin for 10-15 minutes at 4 °C at 14,000 rpm. Remove and SAVE (in a 1.5 ml RNASE-FREE tube) all of the EtOH layer except approximately 50 µl. Spin as above for 5 minutes. Remove the remaining EtOH without disrupting the pellet. Wash pellet with 400 µl of 80% EtOH chilled and spin for 2-5 minutes at 4 °C at 14,000 rpm. Remove EtOH and again spin for 1 minute at 14,000 RPM and remove the remaining 1-5 µl of EtOH by just touching a 20 µl pipette tip to the edge of the drop of EtOH. Air dry with lids open on ice for 5 minutes.

- Resuspend in 4 µl DEPC-Water (Ambion) (250 ng/µl) (markers), (500 ng/µl) (mRNA)
- SAVE 500 ng (2 µl) RNA markers

DAY TWO:

***Continue with 2µg and 5µl of TF-1 mRNA (for biotin-capture)

1st Strand Synthesis

**Add components in the order they are listed.

1.0µl	1.0	µl	tRNA
----	1.0	µl	DEPC-treated water
	4.0	µl	5X 1st Strand Buffer
	2.0	µl	100mM DTT
	0.5	µl	20mM dNTPs (fresh)
4.7 µl	3.7	µl	pED4 NT35 (8/14/98, 300 ng/µl) total 1.1 µg
	0.5	µl	RNAsin
	4.0	µl	Globin mRNA(total 1µg)/MG63 mRNA (total 2µg)
	2.0	µl	Superscript II (Gibco-BRL)
	1.3	µl	Thermoscript RT

V_T = 20 µl

* Incubate at 48°C for 1 hour, 55 °C for 30 minutes
* Add 130 µl of water and 150 µl of phenol/$CHCl_3$/IAA pH 7.9 (Ambion) and "flick" for 0.5 min. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place into new 1.5 ml RNASE-FREE tube.
* Add 125 µl of DEPC-water (Ambion) to the original tube (bottom)) and "flick" for 30 seconds. Spin for 4-6 minute in microcentrifuge at 14,000 rpm. Remove 125 µl aqueous layer with pipette (TOP) and place with the other aqueous layer in the 1.5 ml RNASE-FREE tube.
* Add 25 µl 3M NaOac, pH 4.5 (Autoclaved from media prep) and 625 µl of 100% EtOH. Incubate on dry ice for 5-8 minutes.
* Spin for 10-15 minutes at 4 °C at 14,000 rpm. Remove and SAVE (in a 1.5 ml RNASE-FREE tube) all of the EtOH layer except approximately 50 µl. Spin as above for 5 minutes. Remove the remaining EtOH without disrupting the pellet. Wash pellet with 400 µl of 80% EtOH chilled at –20 °C and spin for 2-5 minutes at 4 °C at 14,000 rpm. Remove EtOH and again spin for 1 minute at 14,000 RPM and remove the remaining 1-5 µl of EtOH by just touching a 20 µl pipette tip to the edge of the drop of EtOH. Air dry with lids open on ice for 5 minutes.

Resuspend in 51.5µl of DEPC-treated water****

0.8% TBE Agarose Gel

****Use only depyrogenated glassware to make the buffer and the gel.**

** Wash your gel box and casting tray with RNASE AWAY.

* Make 1X TBE Buffer, by adding 110 ml of 10X TBE to 1 L of sterile milli -Q water. You may need to make 2 bottles, depending on the size of your gel.
* Using a depyrogenated graduated cylinder measure 120 ml of 1X TBE buffer and pour it into a 500 ml depyrogenated flask. Measure out 1 g of ultra-pure agarose (BI 101) by shaking it into a weigh boat. Add the agarose to the buffer in the flask and swirl.
* Heat the agarose approximately 1.5 minutes in a microwave, or until the agarose is clear. Allow it to cool until you can touch it with your bare hands without it burning, approximately 10 minutes. Add 10 µl of 10 mg/ml ethidium bromide, swirl and pour it into a casting tray. Add comb to the gel and remove all bubble with a pipet tip.
* Wait until it is completely solidified, approximately 20 minutes. In the meantime, add Gel Loading Buffer II (Ambion) in equal volume with your saved samples from the previous three reactions. (Example: if you saved 1 µl then you add 1 µl of dye.) You should have 3 sample of RNA markers at after various reactions. Also, add 0.5 µl of 0.24-9.5 KB RNA Ladder (Gibco-BRL) with 2 µl of water and 2 µl of dye for your gel marker.
* Heat 200 ml of sterile milli-Q water in a 500 ml beaker in the microwave until it boils or set up a 80 °C heat block. Place your gel sample with dye into the water for 5 minutes at 80 °C. Then place them directly on to ice, until you are ready to load them onto the gel.
* Once the gel is hardened place it into the buffer chamber and add buffer to cover it. Load your sample onto the gel. Run the gel at 100 volts for approximately 1 hour, or until the first dye line reaches 2/3ths of the length of the gel. Stop the gel and take a picture.
* You may have lost some mRNA as you progressed through each reaction, show by the decrease in intensity of the stained mRNA.; HOWEVER, the mRNA should all be the same size on the gel. If degradation has occurred, there will be a downshift in the size of the mRNA as the process progressed.

RNASE-treatement

52.0µl	51.5	µl	cDNA (1.1 µg)
	6.0	µl	10X NEB buffer # 2
	2.0	µl	RNase One (Promega, 10 U/µl)
-----	0.5	µl	*E.coli* RNAse H (Epicenter) (10u/µl)

V_T = 60 µl

Incubate at 37°C for 60 minutes

****STOP the 5 µg cDNA Library******

ANNEALING

* JCB Annealing Buffer = 30mM Tris pH 8, 10 mM $MgCl_2$, 300 mM NaCl (made with Ambion Solutions)

60 µl	previous Rxn
30 µl	DEPC-water
10 µl	10X JCB Annealing Buffer
Vt = 100 µl	

Heat to 80 °C for 5 min , remove heating clock and cool until the temperture reaches 37 °C (for 30 minutes).
EtOH precip with glycogen
Resuspend in 10 µl 0.5X TE (110ng/ µl)

2nd Strand Synthesis

2	µl	10X T7 Buffer	
3.6	µl	Water	
10	µl	Annealed cDNA (1.1 µg)	
0.5	µl	20 mM dNTPs	(Epicenter)
0.9	µl	BSA (1 mg/ml)	(NEB)
3	µl	T7 DNA polymerase *dilute to (3 Units/µl)*	(NEB)

V_T = 20 µl

Incubate at 37 °C for 3-5 minutes

Transformation

1 µl (2^{nd})	2nd strand reactions (11 ng) ***diluted (1:5)**
40 µl	Electromax DH10B *E. coli*

V_T = 41 µl

Electropore the transformation reaction at 1.8 volts.
Add 1 ml of SOC media the the cells and transfer to a culture tube.
Grow for 1 hour at 37°C
Plate on to LB + 100 mcg/ml AMP plates (LARGE)--50 µl & 200 µl
Grow around 16 hours

Day Three & Four

*** **Count the colonies and calculate the titer (cfu/µg)**

Culturing for Mini-Preps

- Fill a 96-deep well culture dish with 1 ml of TB with AMP (100µg/ml)
- Pick a single colony using a toothpick and place it into one well. Continue until all wells are inoculated. Remove the toothpicks and cover air pore tape. Grow at least 16 hour overnight (up to 24 hours).

Mini-Preps (Qiagen)

- Spin down plate at 4000 rpm for 10 minutes (Program #7).
- Check for pellet and then pour out media.
- Continue following Qiagen 96-well Turbo Mini-prep protocol

Digests

- Use an U-shaped 96-well culture plate for digests.
- For 105 Rxn at 15 µl/ reaction

210 µl	2 µl	Buffer #3
	5 µl	plasmid
1218 µl	11.6 µl	milli-Q water
63 µl	0.6 µl	Xho I
63 µl	0.6 µl	Pst I
21 µl	0.2 µl	100X BSA
V_T=1575 µl	V_T = 20 µl	

Incubate at 37 °C for 2 hours
Add 3 µl 6X loading dye
Run on gel at 250 volts for 1.5- 2 hours
Stain gel for 10-15 minutes

References

Alexander, D. C., McKnight, T. D., & Williams, B. G. (1984). A simplified and efficient vector-primer cDNA cloining system. Gene, 31(1-3), 79-89.

Bellemare, G., Potvin, C., & Bergeron, D. (1991). High-yield method for directional cDNA library construction. Gene, 98, 231-235.

Carninci, P., Kvam, C., Kitamura, A., Ohsumi, T., Okazaki, Y., Itoh, M., Kamiya, M., Shibata, K., Sasaki, N., Izawa, M., Muramatsu, M., Hayashizaki, Y., & Schneider, C. (1996). High-efficiency full-length cDNA cloning by bionylated CAP trapper. Genomics, 37, 327-336.

Carninci, P., Westover, A., Nishiyama, Y., Ohsumi, T., Itoh, M., Nagaoka, S., Sasaki, N., Okazaki, Y., Muramatsu, M., Schneider, C., & Hayashizaki, Y. (1997). High efficiency selection fo full-lenght cDNA by improved biotinylated cap trapper. DNA Res., 4(1), 61-66.

Edery, I., Chu, L. L., Sonenberg, N., & Pelletier, J. (1995). An efficient strategy to isolate full-length cDNAs based on an mRNA cap retension procedure (CAPture). Mol. Cell. Biol., 15(6), 3363-3371.

Efstratiadis, A., Vournakis, J. N., Donis-Keller, H., Chaconas, G., Dougall, D. K., & Kafatos, F. C. (1977). End labeling of enzymatically decapped mRNA. Nucleic Acids Res., 4(12), 4165-4174.

Fromont-Racine, M., Bertrand, E., Pictet, R., & Grange, T. (1993). A highly sensitive method for mapping the 5' termini of mRNAs. Nucleic Acids Res., 21(7), 1683-1683.

Kato, S., Sekine, S., Oh, S.-W., Kim, N.-S., Umezawa, Y., Abe, N., Yokoyama-Kobayashi, M., & Aoki, T. (1994). Construction of a human full-length cDNA bank. Gene, 150, 243-250.

Liu, X., & Gorovsky, M. A. (1993). Mapping the 5' and 3' ends of Tetrahymena thermophila mRNA using RNA ligase mediated amplification of cDNA ends (PLM-RACE). Nucleic Acids Res., 21(21), 4954-4960.

Maruyama, K., & Sugano, S. (1994). Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. Gene, 138, 171-174.

Okayama, H., & Berg, P. (1982). High-efficiency cloning of full-length cDNA. Mol. Cell. Biol., 2(2), 161-170.

Shinshi, H., Miwa, M., Kato, K., Noguchi, M., & Matsushima, T. (1976a). A novel phosphodiesterase from cultured tobacco cells. Biochemistry, 15, 2185-2190.

Shinshi, H., Miwa, M., Sugimura, T., Shimotohno, K., & Miura, K.-I. (1976b). Enzyme cleaving the 5'-terminal methylated blocked structure of messenger RNA. FEBS Lett., 65(2), 254-257.

Soares, M. B., Bonaldo, M. F., Jelene, P., Su, L., Lawton, L., & Efstratiadis, A. (1994). Construction and characterization of a normalized cDNA library. Proc Natl Acad Sci U S A, 91(20), 9228-9232.

Tessier, D. C., Brousseau, R., & Vernet, T. (1986). Ligation of single-stranded oligodeoxyribonucleotides by T4 RNA ligase. Analy. Biochem., 158, 171-178.

Yokoyama-Kobayashi, M., & Kota, S. (1993). Rocombinant f1 phage particles can transfect monkey COS-7 cells by DEAE detran method. Biochem. Biophys. Res. Comm., 192, 935-939.

Description of Tables

Table 1 shows the results of making a cDNA library of rabbit globin mRNA using the PAVE method of the present invention.

Table 2 shows the results of making cDNA libraries from a variety of mRNA sources using both "conventional" methods and the PAVE method of the present invention. The "conventional" method employed a kit obtained from GIBCO/BRL and utilized a 3' oligo-dT primer and SauI adaptors.

Table 3 shows a number of parameters of the T4 RNA ligase reaction that may be modified to obtain optimal efficiency of the reaction. The most preferred reaction conditions include performing the reaction at room temperature overnight (or 16 hours); using an acceptor/donor ratio that is the same as that obtained from reacting 2 μg mRNA (average size 1.5 kb) with 175 pmoles of a 27-residue RNA tag; and performing the reaction in RNAse-free Tris $MgCl_2$ buffer with tRNA, DTT, and 5.8nM ATP added.

Table 1. Analysis of cDNA library made from rabbit globin mRNA

	Number of Colonies	Percentage
Total Positives [a]	385	100%
Full-length [b]	292	75.8%
3'-only [c]	75	19.5%
5'-only [d]	18	4.7%

a. Duplicate filters were lifted from one plate and hybridized to two labeled oligonucleotide probes complementary to 5' and 3' ends of rabbit β-globin mRNA. The total positives were counted.

b. Full-length clones were double positives to 5' and 3' probes.

c. Clones hybridized only to 3'-end probes.

d. Clones hybridized only to 5'-end probes.

TABLE 2.

cDNA Library Comparison

	Type	Tissue/Cells	100 % FL	98.5% FL	# Sequenced	#mRNA cds	% Flipped	Median % FL	Correct Size	Average FL Siz
	Conventional	HT1080	42%	48%	342	91	1.3%	95%	78%	601bp
	Conventional	Thymus	19%	23%	4263	663	0.5%	58%	58%	2003bp
	Conventional	WERI-RB	23%	26%	4021	715	0.3%	63%	50%	1275bp
	Pave	HT1080	64%	67%	206	49	0.1%	93%	81%	993bp
	Pave	Thymus	50%	50%	40	20	0.0%	98%	N/A	562bp
	Pave	WERI-RB	34%	38%	278	63	0.0%	90%	N/A	956bp

Definitions of Data Table Categories:

Type = Two types of cDNA libraries were analyzed in this study. "Conventional" refers to libraries that are constructed with a 3' oligo dT primer. "PAVE" refers to 5'-directed cDNA library construction technology.

100% FL = Represents the percentage of clones that contain 100% or greater 5' sequence relative to their respective GenBank record

98.5% FL = Represents the percentage of clones that contain 98.5% or greater 5' sequence relative to their respective GenBank record

Median % FL = Represents the median full-length value of all the clones analyzed.

Correct Size = Represents the percentage of clones that were 100% FL or greater than size matched after restriction digestion analysis

Average FL Size = Represents the average size of the clones that were 100% full-length based on restriction enzyme digestion analysis.

TABLE 3

Optimization of RNA-RNA ligation by T4 RNA ligase

1. Effect of Temperature: 4 °C, O/N; 16 °C O/N; Room Temperature O/N; 37 °C, O/N; 37 °C, 3 hrs

2. Time Courses at Suitable Temperature: 0.5, 2, 4, 8, 16, 24 hrs

3. Effect of Denaturants: DMSO: 10%, 20%, 30%, 40%
   Urea: 0.5 M, 1M, 2M, 3M, 4M
   Formamide: 5%, 10%, 20%, 40%

4. Effect of Accepter/Donor Ratio: 1, 10, 20, 50, 100, 200

5. Effect of PEG: 5%, 10%, 15%, 20%, 25%

6. Effect of Buffers (?): Glycylglycine, HEPES or Tris

7. Effect of Inorganic Pyrophosphatase (PPi is inhibitory, but Pi is not!!)

8. Effect of HCC(hexamine cobalt chloride): 0.5 mM, 1 mM, 2 mM, 5 mM, 10 mM.

9. Effect of Single-Stranded RNA Binding Proteins (*i.e.* T4 gene 32 protein)

What is claimed is:

1. A method for preparing a modified mRNA molecule which comprises ligating a tag comprising at least one ribonucleotide residue to the 5′ end of one or more mRNA molecules, wherein the tag does not contain deoxyribonucleotide residues.

2. The method of claim 1 further comprising a prior step of treating at least one mRNA molecule with pyrophosphatase so that the 7-methylguanosine (7mG) cap is removed from the 5′ end of at least one mRNA molecule.

3. The method of claim 2 wherein the pyrophosphatase is tobacco acid pyrophosphatase.

4. The method of claim 1 further comprising a prior step of treating at least one mRNA molecule with phosphatase so that the 5′ phosphate is removed from at least one mRNA molecule not having a 7-methylguanosine (7mG) cap.

5. The method of claim 4 wherein the phosphatase is selected from the group consisting of HK phosphatase and BA phosphatase.

6. The method of claim 1 wherein the tag further comprises a biotin residue.

7. The method of claim 1 wherein the tag has the following ribonucleotide sequence: 5′-ACUAGUGACCAGCUGAUACGCCUCAAA-3′

8. The method of claim 1 wherein the ligation reaction is performed using T4 RNA ligase.

9. The method of claim 1 wherein the ligation reaction is performed at room temperature overnight.

10. The method of claim 1 wherein the ligation reaction is performed in the presence of tRNA molecules.

11. The method of claim 1 wherein the ligation reaction is performed in an ATP concentration selected from the group consisting of: 2 nM, 3 nM, 4 nM, 4.5 nM, 5 nM, 5.5 nM, 5.8 nM, 6 nM, 6.5 nM, 7 nM, 7.5 nM, 8 nM, 9 nM, and 10 nM.

12. The method of claim 11 wherein the ATP concentration is 5.8 nM.

13. A modified mRNA molecule produced according to the method of claim 1.

14. A method for preparing at least one vector-primer molecule which comprises contacting at least one primer with at least one vector molecule so that at least one complementary base-pair is formed between the primer and the vector molecule.

15. The method of claim 14 wherein the vector is selected from the group consisting of pED6dpc2, pED6dpc4, pNOTs, and pAVE1.

16. The method of claim 14 wherein at least one primer has a nucleotide sequence selected from those shown as "3' linker" and "5' linker" in Figures 7 and 8.

17. The method of claim 14 further comprising a subsequent step of ligating at least one primer to at least one vector molecule.

18. The method of claim 17 wherein the ligation reaction is performed with T4 DNA ligase.

19. A vector-primer molecule produced according to the method of claim 14.

20. A method for preparing a cDNA library comprising the steps of:

(a) ligating a tag comprising at least one ribonucleotide residue to the 5' end of one or more mRNA molecules, wherein the tag does not contain deoxyribonucleotide residues;

(b) contacting the products of step (a) with a vector-primer molecule so that at least one complementary base-pair is formed between at least one product of step (a) and the vector-primer molecule.

21. The method of claim 20 further comprising a subsequent RNAse digestion step.

22. The method of claim 20 further comprising a subsequent DNA polymerase second-strand synthesis step.

23. The method of claim 22 wherein the DNA polymerase is selected from the group consisting of T4, T7, Pfu, and SEQUENASE DNA polymerases.

24. The method of claim 22 wherein the DNA polymerase reaction is perfromed for a time period selected from the group consisting of: 1 minute, 2.5 minutes, 5 minutes, 7.5 minutes, 10 minutes, 20 minutes, 30 minutes, or 60 minutes.

25. The method of claim 24 wherein the DNA polymerase reaction is performed for 5 minutes.

26. The method of claim 20 further comprising a subsequent step comprising transforming host cells with the products of step (b) of claim 20.

27. The method of claim 26 wherein the host cells are transformed with the products of step (b) of claim 20 without a DNA polymerase second-strand synthesis step having been performed.

28. The method of claim 26 wherein the host cells are transformed with the products of step (b) of claim 20 without a DNA ligase step having been performed.

29. A cDNA library comprising cDNA molecules produced according to the method of claim 20.

30. The method of claim 20, wherein the mRNA molecules are human mRNA molecules.

31. The method of claim 20, wherein the mRNA molecules are mammalian mRNA molecules.

32. The method of claim 20, wherein the mRNA molecules are mRNA molecules extracted from a species of plant.

33. The cDNA library of claim 29, wherein the mRNA molecules of claim 20 are human mRNA molecules.

FIGURE 1

Labeling of Full-length mRNA With An RNA Tag

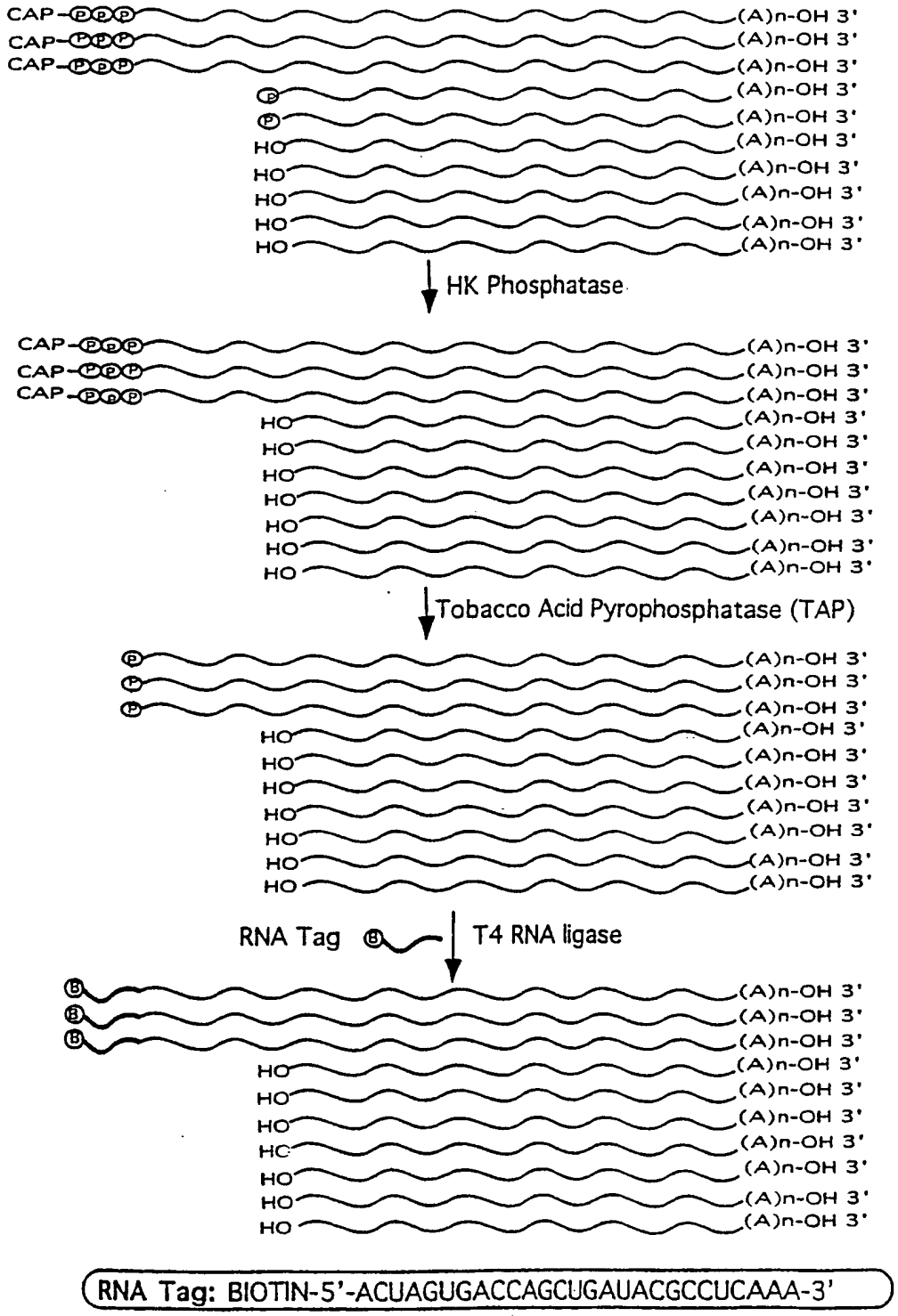


FIGURE 2

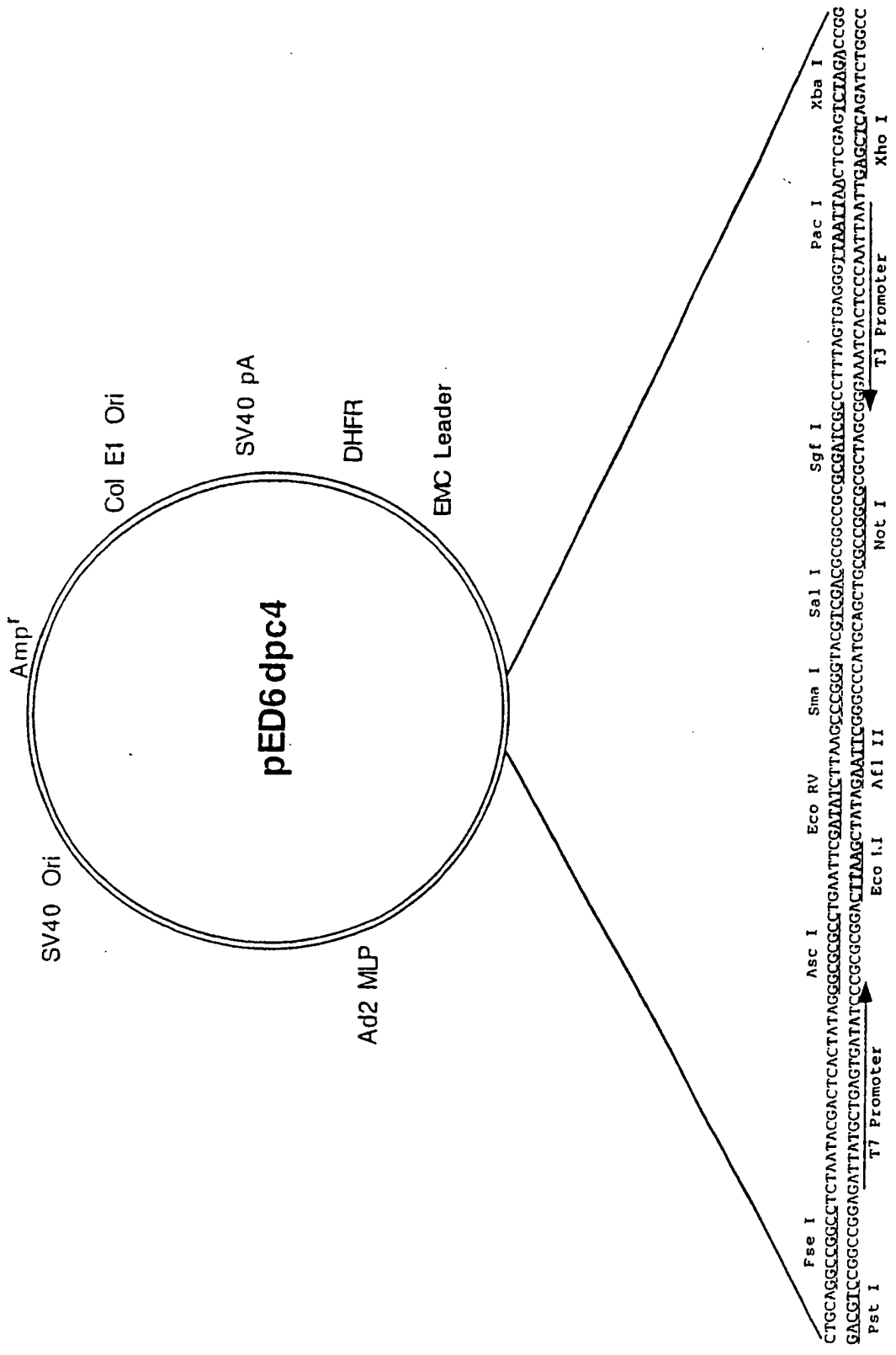

pED6dpc4
Amp^r
Col E1 Ori
SV40 pA
DHFR
EMC Leader
SV40 Ori
Ad2 MLP
Fse I
Asc I
Eco RV
Sma I
Sal I
Sgf I
Pac I
Xba I
Pst I
T7 Promoter
Eco 1.1
Afl II
Not I
T3 Promoter
Xho I

FIGURE 3

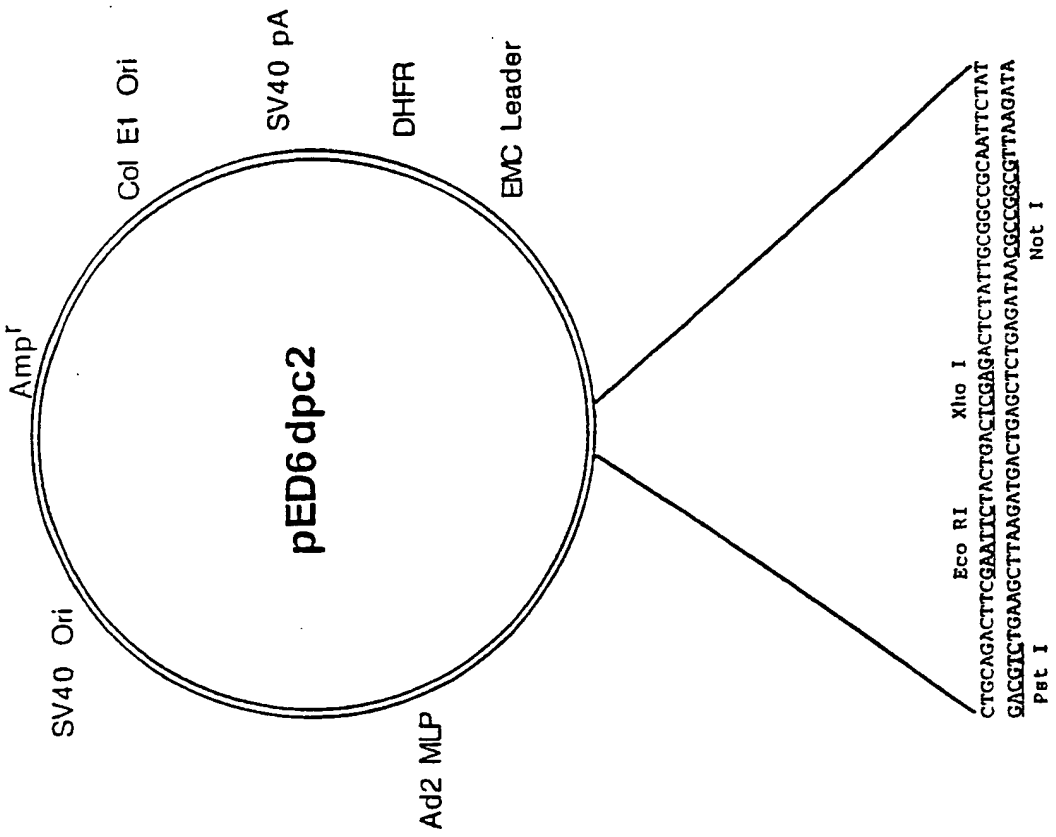
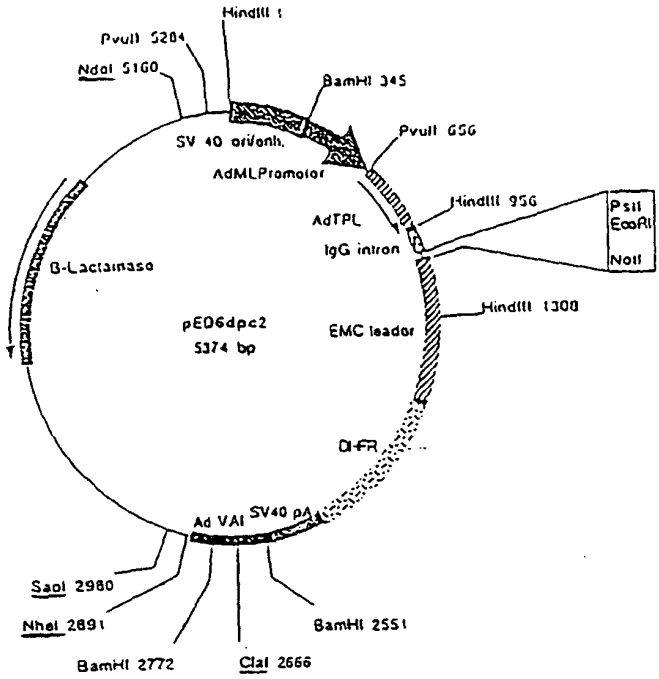

pED6dpc2
Amp^r
Col E1 Ori
SV40 pA
DHFR
EMC Leader
SV40 Ori
Ad2 MLP
Eco RI
Xho I
Not I
Pst I
CTGCAGACTTCGAATTCTACTGACTCGAGACTCTATTGCGGCCGCAATTCTAT
GACGTCTGAAGCTTAAGATGACTGAGCTCTGAGATAACGCCGGCGTTAAGATA

FIGURE 4

FIGURE 5 pED6dpC2

RESTRICTION AND FUNCTIONAL MAP
OF THE pED6dpc2 EXPRESSION PLASMID



Plasmid Name: pED6dpc2
Plasmid Size: 5374 bp

Comments: The origin, function, and position of the various elements of the pED6dpc2 expression plasmid are provided below. The various nucleotide (nt) positions within the plasmid are given relative to the 5' end of the SV40 enhancer segment, the first nt of which was assigned as Position 1.

DiscoverEase™ cDNAs are cloned between EcoRI and NotI.
ClaI, NheI, SapI, and NdeI are unique sites in the expression plasmid.

SV40 enhancer (nt 1-345): This fragment originated from the SV40 genome. It contains the SV40 origin of replication and transcriptional enhancer. The SV40 enhancer sequence increases the level of transcription from the adenovirus 2 (Ad2) major late promoter.

Ad2 MLP (nt 364-656): This fragment contains the Ad2 major late promotor (MLP) from XhoI to PvuII.

Ad2 TPL (nt 657-796): This fragment represents a cDNA copy of the majority of the tripartite leader present on all late Ad2 mRNAs.

Hybrid intron (nt 797-1059): The hybrid intervening sequence contains a 5' splice from the Adenovirus tripartite leader and a 3' splice from a murine IgG gene.

Polylinker (nt 1059-1093): The DiscoverEase™ cDNAs are cloned into the EcoRI-NotI site. The 5' end of the cDNAs contains a SfiI site.

EMCV Leader (nt 1104-1649): This sequence is derived from the encephalomyocarditis virus (EMCV) RNA. This sequence allows ribosomes to initiate translation internally, resulting in a more efficient translation of the DHFR gene.

Mouse DHFR cDNA (nt 1650-2317): A selectable marker in Chinese hamster ovary cells.

SV40 polyadenylation site (nt 2318-2550): This fragment contains the polyadenylation site from the SV40 early region.

Ad2 VAI gene (nt 2551-2905): This fragment is derived from the Ad2 genome and encodes the virus-associated RNA I.

pUC 19 backbone (nt 2906-5374): This fragment includes the Col E1 origin of replication which allows replication of the plasmid in *E. coli*, and the beta-lactamase gene (nt 3913-4708) which confers ampicillin resistance and is used as a selectable marker in the propagation of the plasmid in *E. coli*.

FIGURE 6

The following is the sequence alignment of pED6dpc2 and pED6dpc4.

```
dpc2    1 AAGCTTTTTGCAAAAGCCTAGGCCTCCAAAAAAGCCTCCTCACTACTTCT 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
dpc4    1 AAGCTTTTTGCAAAAGCCTAGGCCTCCAAAAAAGCCTCCTCACTACTTCT 50

       51 GGAATAGCTCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAAAAA 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
       51 GGAATAGCTCAGAGGCCGAGGCGGCCTCGGCCTCTGCATAAATAAAAAAA 100

      101 ATTAGTCAGCCATGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGG 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      101 ATTAGTCAGCCATGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGG 150

      151 CGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCTGACTAATTGAGAT 200
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      151 CGGGATGGGCGGAGTTAGGGGCGGGACTATGGTTGCTGACTAATTGAGAT 200

      201 GCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACAC 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      201 GCATGCTTTGCATACTTCTGCCTGCTGGGGAGCCTGGGGACTTTCCACAC 250

      251 CTGGTTGCTGACTAATTGAGATGCATGCTTTGCATACTTCTGCCTGCTGG 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      251 CTGGTTGCTGACTAATTGAGATGCATGCTTTGCATACTTCTGCCTGCTGG 300

      301 GGAGCCTGGGGACTTTCCACACCCTAACTGACACACATTCCACAGGATCC 350
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      301 GGAGCCTGGGGACTTTCCACACCCTAACTGACACACATTCCACAGGATCC 350

      351 GGTCGCGCGAATTTCGAGCGGTGTTCCGCGGTCCTCCTCGTATAGAAACT 400
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      351 GGTCGCGCGAATTTCGAGCGGTGTTCCGCGGTCCTCCTCGTATAGAAACT 400

      401 CGGACCACTCTGAGACGAAGGCTCGCGTCCAGGCCAGCACGAAGGAGGCT 450
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      401 CGGACCACTCTGAGACGAAGGCTCGCGTCCAGGCCAGCACGAAGGAGGCT 450

      451 AAGTGGGAGGGGTAGCGGTCGTTGTCCACTAGGGGGTCCACTCGCTCCAG 500
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      451 AAGTGGGAGGGGTAGCGGTCGTTGTCCACTAGGGGGTCCACTCGCTCCAG 500

      501 GGTGTGAAGACACATGTCGCCCTCTTCGGCATCAAGGAAGGTGATTGGTT 550
          |-|||||||||||||||||||||||||||||||||||||||||||||||||
      501 GGTGTGAAGACACATGTCGCCCTCTTCGGCATCAAGGAAGGTGATTGGAA 550
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FIGURE 6 (CONTINUED)

```
551 TATAGGTGTAGGCCACGTGACCGGGTGTTCCTGAAGGGGGGCTATAAAAG 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
551 TATAGGTGTAGGCCACGTGACCGGGTGTTCCTGAAGGGGGGCTATAAAAG 600

601 GGGGTGGGGGCGCGTTCGTCCTCACTCTCTTCCGCATCGCTGTCTGCGAG 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
601 GGGGTGGGGGCGCGTTCGTCCTCACTCTCTTCCGCATCGCTGTCTGCGAG 650

651 GGCCAGCTGTTGGGCTCGCGGTTGAGGACAAACTCTTCGCGGTCTTTCCA 700
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
651 GGCCAGCTGTTGGGCTCGCGGTTGAGGACAAACTCTTCGCGGTCTTTCCA 700

701 GTACTCTTGGATCGGAAACCCGTCGGCCTCCGAACGGTACTCCGCCACCG 750
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
701 GTACTCTTGGATCGGAAACCCGTCGGCCTCCGAACGGTACTCCGCCACCG 750

751 AGGGACCTGAGCGAGTCCGCATCGACCGGATCGGAAAACCTCTCGACTGT 800
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
751 AGGGACCTGAGCGAGTCCGCATCGACCGGATCGGAAAACCTCTCGACTGT 800

801 TGGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAAGATT 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
801 TGGGGTGAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAAGATT 850

851 GTCAGTTTCCAAAAACGAGGAGGATTTGATATTCACCTGGCCCGCGGTGA 900
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
851 GTCAGTTTCCAAAAACGAGGAGGATTTGATATTCACCTGGCCCGCGGTGA 900

901 TGCCTTTGAGGGTGGCCGCGTCCATCTGGTCAGAAAAGACAATCTTTTTG 950
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
901 TGCCTTTGAGGGTGGCCGCGTCCATCTGGTCAGAAAAGACAATCTTTTTG 950

951 TTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGT 1000
    ||||||||||||||||||||||||||||||||||||||||||||||||||||
951 TTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGT 1000

1001 GACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGT 1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
1001 GACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGT 1050

1051 CCAACTGCA.....................................Gact 1063
     |||||||||                                     || |
1051 CCAACTGCAGGCCGGCCtctaatacgactcactatagGGCGCGCCtgaat 1100

1064 tcGAATTCt..................................... 1072
     ||||  |||
1101 tcGATATCttaagCCCGGGtacGTCGACgcggccgcGCGATCGCcctttta 1150
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FIGURE 6 (CONTINUED)

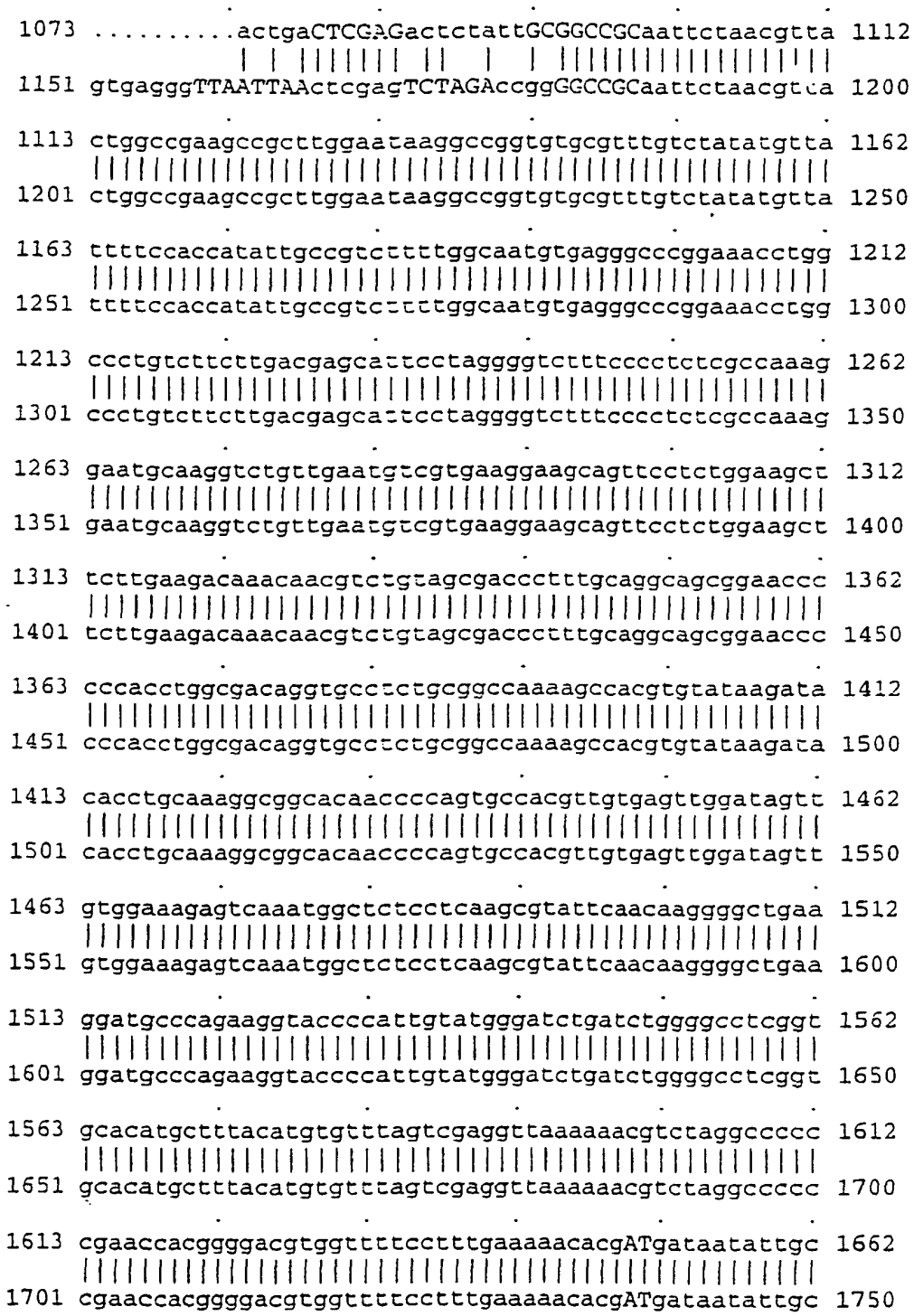

FIGURE 6 (CONTINUED)

```
1663 cacaaccatggttcgaccattgaactgcatcgtcgccgtgtcccAAAATA 1712
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1751 cacaaccatggttcgaccattgaactgcatcgtcgccgtgtcccAAAATA 1800

1713 TGGGGATTGGCAAGAACGGAGACCTACCCTGGCCTCCGCTCAGGAACGAG 1762
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1801 TGGGGATTGGCAAGAACGGAGACCTACCCTGGCCTCCGCTCAGGAACGAG 1850

1763 TTCAAGTACTTCCAAAGAATGACCACAACCTCTTCAGTGGAAGGTAAACA 1812
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1851 TTCAAGTACTTCCAAAGAATGACCACAACCTCTTCAGTGGAAGGTAAACA 1900

1813 GAATCTGGTGATTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGA 1862
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1901 GAATCTGGTGATTATGGGTAGGAAAACCTGGTTCTCCATTCCTGAGAAGA 1950

1863 ATCGACCTTTAAAGGACAGAATTAATATAGTTCTCAGTAGAGAACTCAAA 1912
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1951 ATCGACCTTTAAAGGACAGAATTAATATAGTTCTCAGTAGAGAACTCAAA 2000

1913 GAACCACCACGAGGAGCTCATTTTCTTGCCAAAAGTTTGGATGATGCCTT 1962
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2001 GAACCACCACGAGGAGCTCATTTTCTTGCCAAAAGTTTGGATGATGCCTT 2050

1963 AAGACTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGA 2012
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2051 AAGACTTATTGAACAACCGGAATTGGCAAGTAAAGTAGACATGGTTTGGA 2100

2013 TAGTCGGAGGCAGTTCTGTTTACCAGGAAGCCATGAATCAACCAGGCCAC 2062
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2101 TAGTCGGAGGCAGTTCTGTTTACCAGGAAGCCATGAATCAACCAGGCCAC 2150

2063 CTCAGACTCTTTGTGACAAGGATCATGCAGGAATTTGAAAGTGACACGTT 2112
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2151 CTCAGACTCTTTGTGACAAGGATCATGCAGGAATTTGAAAGTGACACGTT 2200

2113 TTTCCCAGAAATTGATTTGGGGAAATATAAACTTCTCCCAGAATACCCAG 2162
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2201 TTTCCCAGAAATTGATTTGGGGAAATATAAACTTCTCCCAGAATACCCAG 2250

2163 GCGTCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAGTTTGAA 2212
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2251 GCGTCCTCTCTGAGGTCCAGGAGGAAAAAGGCATCAAGTATAAGTTTGAA 2300

2213 GTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGCTCC 2262
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2301 GTCTACGAGAAGAAAGACTAACAGGAAGATGCTTTCAAGTTCTCTGCTCC 2350
```

FIGURE 6 (CONTINUED)

```
2263 CCTCCTAAAGCTATGCATTTTTTATAAGACCATGGGACTTTTGCTGGCTT 2312
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2351 CCTCCTAAAGCTATGCATTTTTTATAAGACCATGGGACTTTTGCTGGCTT 2400

2313 TAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAA 2362
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2401 TAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAA 2450

2363 AAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTG 2412
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2451 AAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTG 2500

2413 TTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAAT 2462
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2501 TTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAAT 2550

2463 AGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTG 2512
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2551 AGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTG 2600

2513 TGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCCCGGCC 2562
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2601 TGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGGATCCCCGGCC 2650

2563 AACGGTCTGGTGACCCGGCTGCGAGAGCTCGGTGTACCTGAGACGCGAGT 2612
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2651 AACGGTCTGGTGACCCGGCTGCGAGAGCTCGGTGTACCTGAGACGCGAGT 2700

2613 AAGCCCTTGAGTCAAAGACGTAGTCGTTGCAAGTCCGCACCAGGTACTGA 2662
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2701 AAGCCCTTGAGTCAAAGACGTAGTCGTTGCAAGTCCGCACCAGGTACTGA 2750

2663 TCATCGATGCTAGACCGTGCAAAAGGAGAGCCTGTAAGCGGGCACTCTTC 2712
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2751 TCATCGATGCTAGACCGTGCAAAAGGAGAGCCTGTAAGCGGGCACTCTTC 2800

2713 CGTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGGACGACCGGGGT 2762
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2801 CGTGGTCTGGTGGATAAATTCGCAAGGGTATCATGGCGGACGACCGGGGT 2850

2763 TCGAACCCCGGATCCGGCCGTCCGCCGTGATCCATCCGGTTACCGCCCGC 2812
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2851 TCGAACCCCGGATCCGGCCGTCCGCCGTGATCCATCCGGTTACCGCCCGC 2900

2813 GTGTCGAACCCAGGTGTGCGACGTCAGACAACGGGGGAGCGCTCCTTTTG 2862
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2901 GTGTCGAACCCAGGTGTGCGACGTCAGACAACGGGGGAGCGCTCCTTTTG 2950
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FIGURE 4 (CONTINUED)

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2863 GCTTCCTTCCAGGCGCGGCGGCTGCTGCGCTAGCTTTTTTGGCGAGCTCG 2912
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2951 GCTTCCTTCCAGGCGCGGCGGCTGCTGCGCTAGCTTTTTTGGCGAGCTCG 3000

2913 AATTAATTCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG 2962
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3001 AATTAATTCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG 3050

2963 CGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGT 3012
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3051 CGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGT 3100

3013 CGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGT 3062
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3101 CGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGT 3150

3063 TATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGC 3112
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3151 TATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGC 3200

3113 CAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA 3162
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3201 CAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA 3250

3163 TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA 3212
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3251 TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA 3300

3213 GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGA 3262
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3301 GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGA 3350

3263 AGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCT 3312
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3351 AGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCT 3400

3313 GTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCT 3362
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3401 GTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCT 3450

3363 GTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTG 3412
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3451 GTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTG 3500

3413 CACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCG 3462
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3501 CACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCG 3550
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FIGURE 6 (CONTINUED)

```
3463 TCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCA 3512
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3551 TCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCA 3600

3513 CTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC 3562
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3601 CTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC 3650

3563 TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTAT 3612
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3651 TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTAT 3700

3613 CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTT 3662
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3701 CTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTT 3750

3663 GATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAG 3712
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3751 GATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAG 3800

3713 CAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT 3762
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3801 CAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT 3850

3763 TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTT 3812
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3851 TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTT 3900

3813 TGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAA 3862
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3901 TGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAA 3950

3863 AAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGA 3912
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3951 AAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGA 4000

3913 CAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT 3962
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4001 CAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT 4050

3963 TTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATA 4012
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4051 TTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATA 4100

4013 CGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCC 4062
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4101 CGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCC 4150
```

FIGURE 6 (CONTINUED)

```
4063 ACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGG 4112
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4151 ACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGG 4200

4113 CCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATT 4162
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4201 CCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATT 4250

4163 AATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCG 4212
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4251 AATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCG 4300

4213 CAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTG 4262
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4301 CAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTG 4350

4263 GTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGA 4312
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4351 GTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGA 4400

4313 TCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT 4362
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4401 TCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT 4450

4363 TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCAC 4412
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4451 TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCAC 4500

4413 TGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACT 4462
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4501 TGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACT 4550

4463 GGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAG 4512
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4551 GGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAG 4600

4513 TTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAA 4562
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4601 TTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAA 4650

4563 CTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCA 4612
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4651 CTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCA 4700

4613 AGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC 4662
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4701 AGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC 4750
```

FIGURE 6 (CONTINUED)

```
4663 CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAA 4712
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4751 CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAA 4800

4713 AAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAA 4762
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4801 AAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAA 4850

4763 TGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCA 4812
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4851 TGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCA 4900

4813 GGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA 4862
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4901 GGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA 4950

4863 AACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC 4912
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
4951 AACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC 5000

4913 TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCAC 4962
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5001 TAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCAC 5050

4963 GAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGAC 5012
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5051 GAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGAC 5100

5013 ACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGG 5062
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5101 ACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGG 5150

5063 AGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGG 5112
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5151 AGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGG 5200

5113 CTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA 5162
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5201 CTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATA 5250

5163 TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGG 5212
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5251 TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGG 5300

5213 CGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCG 5262
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
5301 CGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCG 5350
```

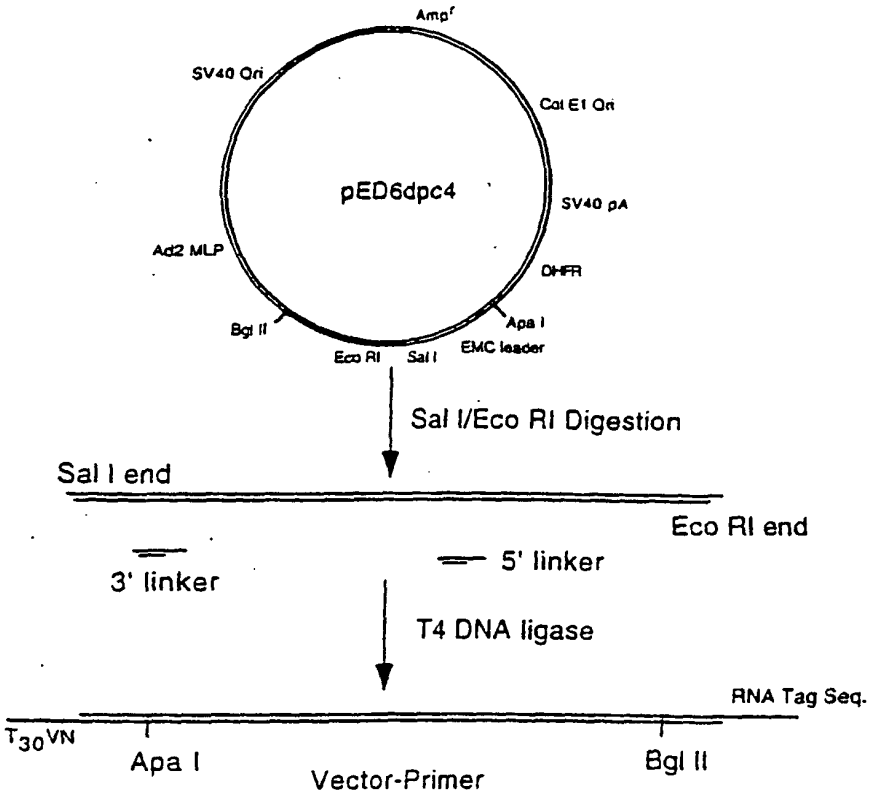
FIGURE 6 (CONTINUED)

```
5263 GGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGC 5312
     ||||||||||||||||||||||||||||||||||||||||||||||||||
5351 GGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGC 5400

5313 GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACG 5362
     ||||||||||||||||||||||||||||||||||||||||||||||||||
5401 GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACG 5450

5363 ACGGCCAGTGCC 5374
     ||||||||||||
5451 ACGGCCAGTGCC 5462
```

FIGURE 7



(Quality assurance with Apa I and Bgl II Digestion)

3' linker

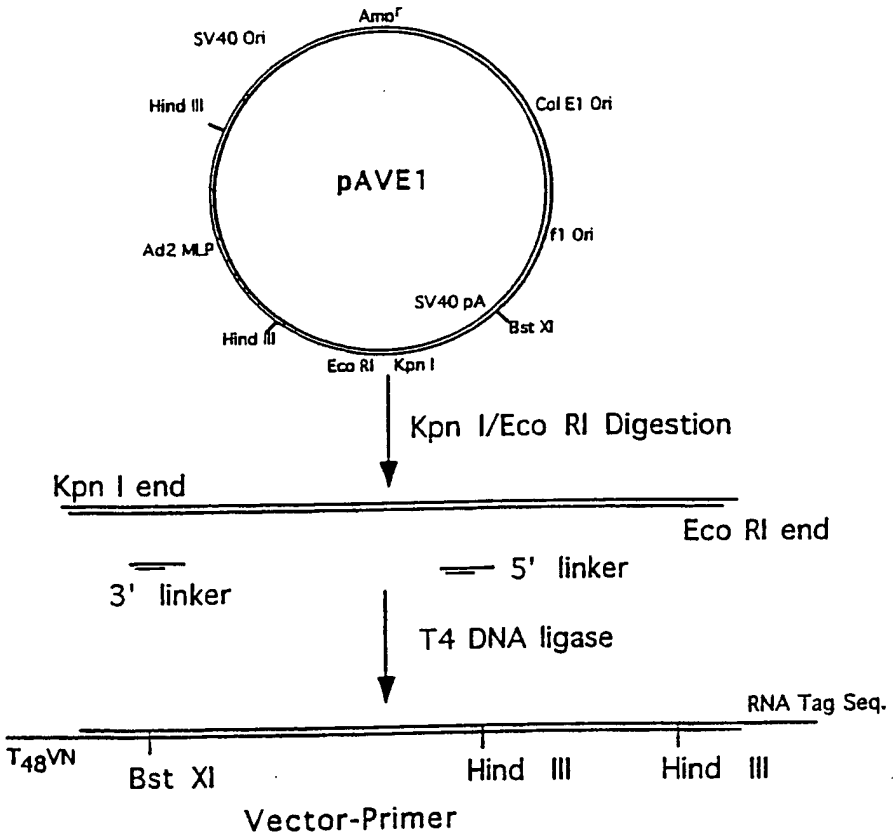
```
          5'-CTAATCTGATCCGCTAGTGGTAC-3'
3'-NV(T)30GATTAGACTAGGCGATCACCATGAGCT-5'
                                  V=A,C,G N=A,C,G,T
```

5' linker

```
                                  RNA Tag Sequence
5'-AATTCGAGTGAACACTCGAGCTCACTAGTGACCAGCTGATACGCCTCAAA-3'
   3'-GCTCACTTGTGAGCTCGAG-5'
```

FIGURE 8

Preparation of Primers-Attached-Vector



(Quality assurance with Hind III and Bst XI Digestion)

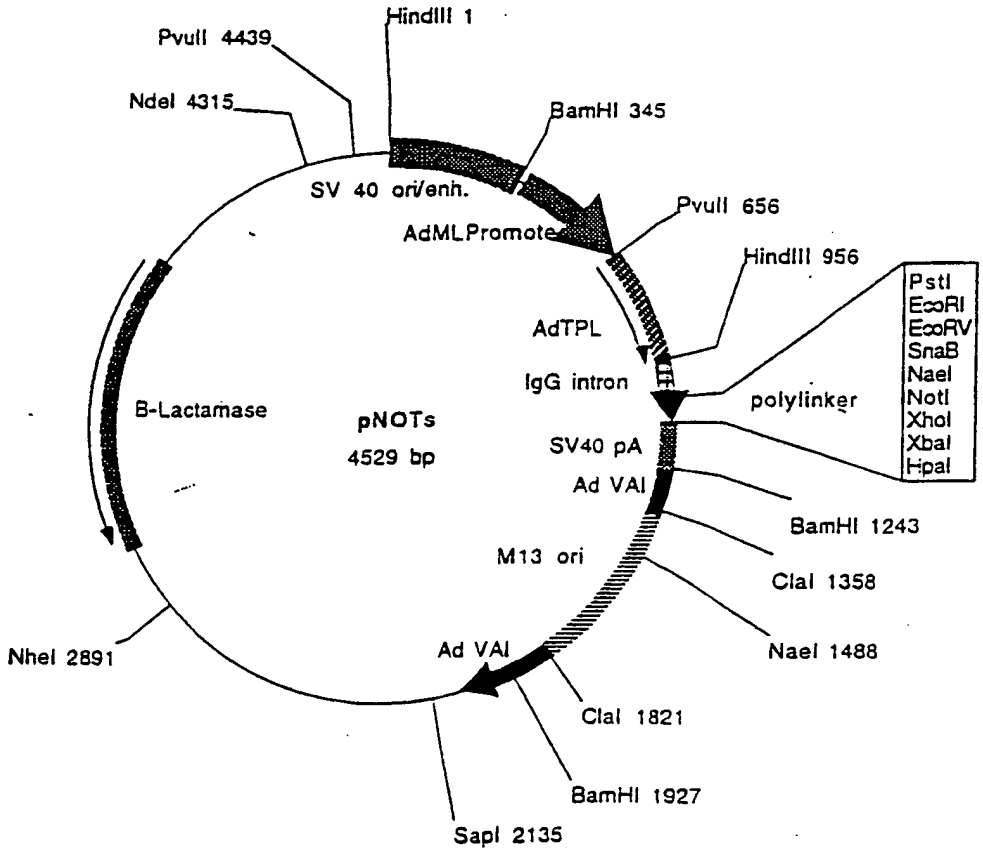
3' linker

```
          5'-CTAATCTGATCCGCTAGTGGTAC-3'
3'-NV(T)48GATTAGACTAGGCGATCAC-5'        V=A,C,G N=A,C,G,T
```

5' linker

```
                                        RNA Tag Sequence
5'-AATTCGAGTGAACACTCGAGCTCACTAGTGACCAGCTGATACGCCTCAAA-3'
   3'-GCTCACTTGTGAGCTCGAG-5'
```

FIGURE 9



Plasmid name: pNOTs
Plasmid size: 4529 bp

946-958

Comments/References: pNOTs is a derivative of pMT2 (Kaufman et al, 1989. Mol.Cell.Biol.9: ~~1[illegible]~~). DHFR was deleted and a new polylinker was inserted between EcoRI and HpaI. M13 origin of replication was inserted in the ClaI site. SST cDNAs are cloned between EcoRI and NotI

FIGURE 10

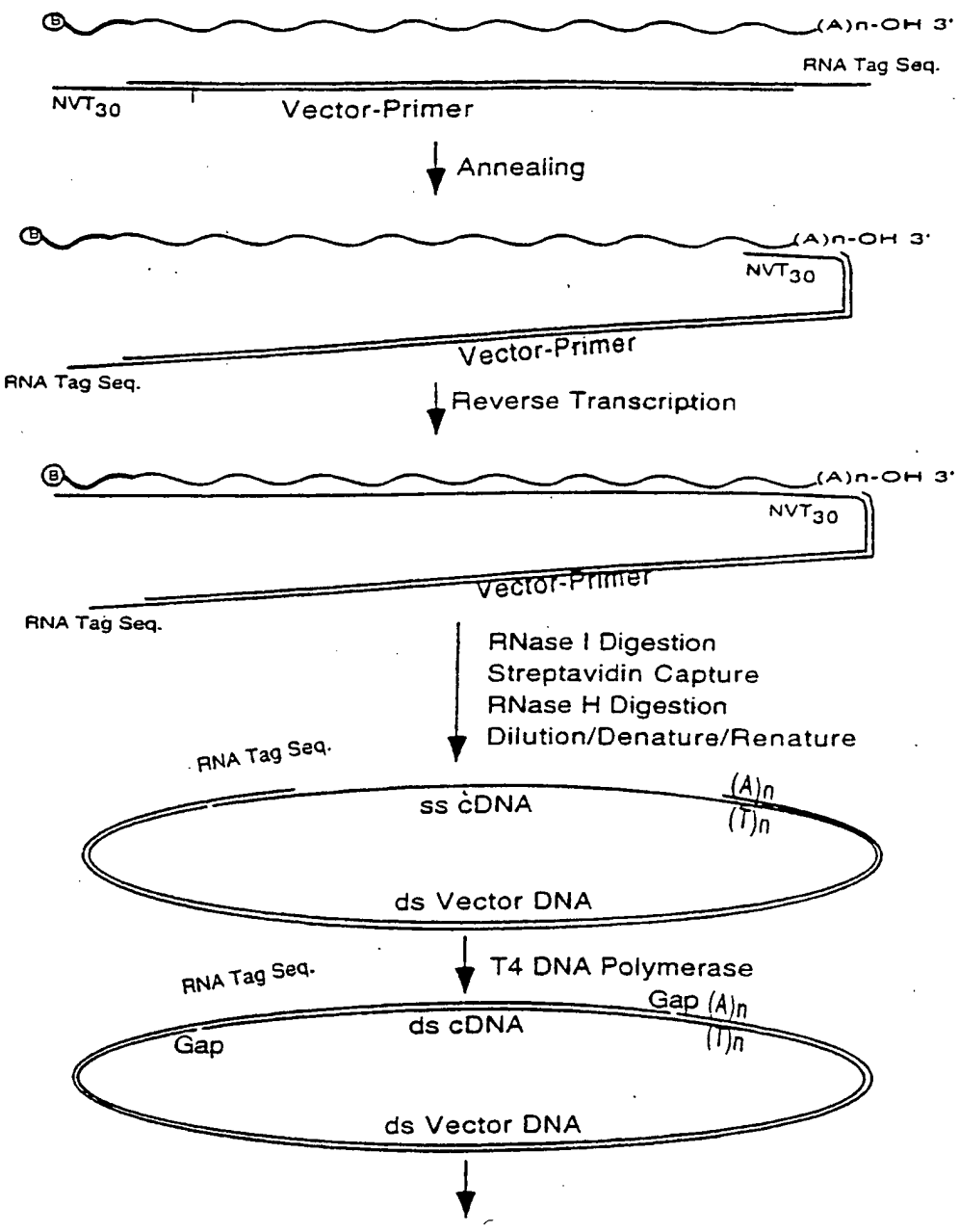
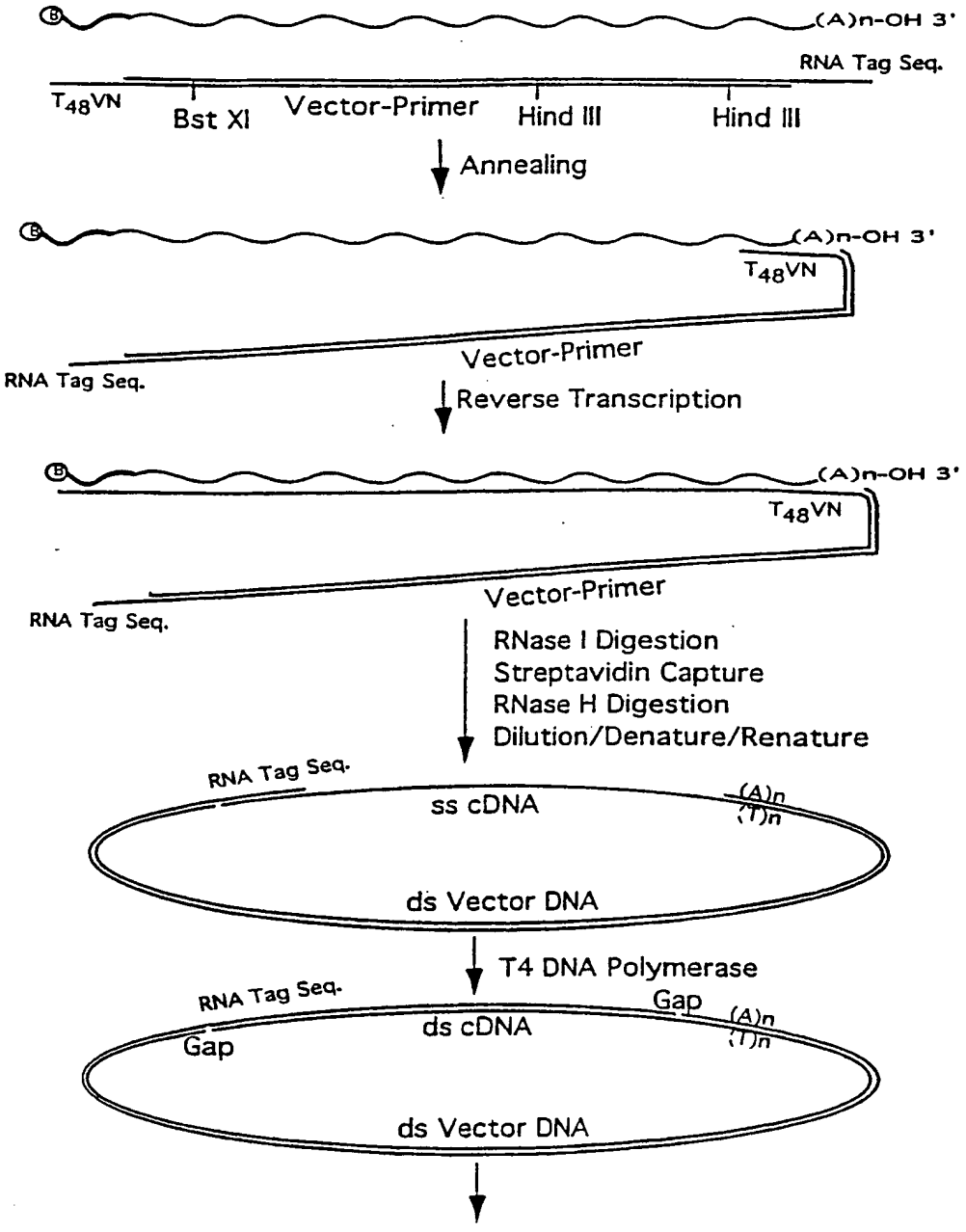
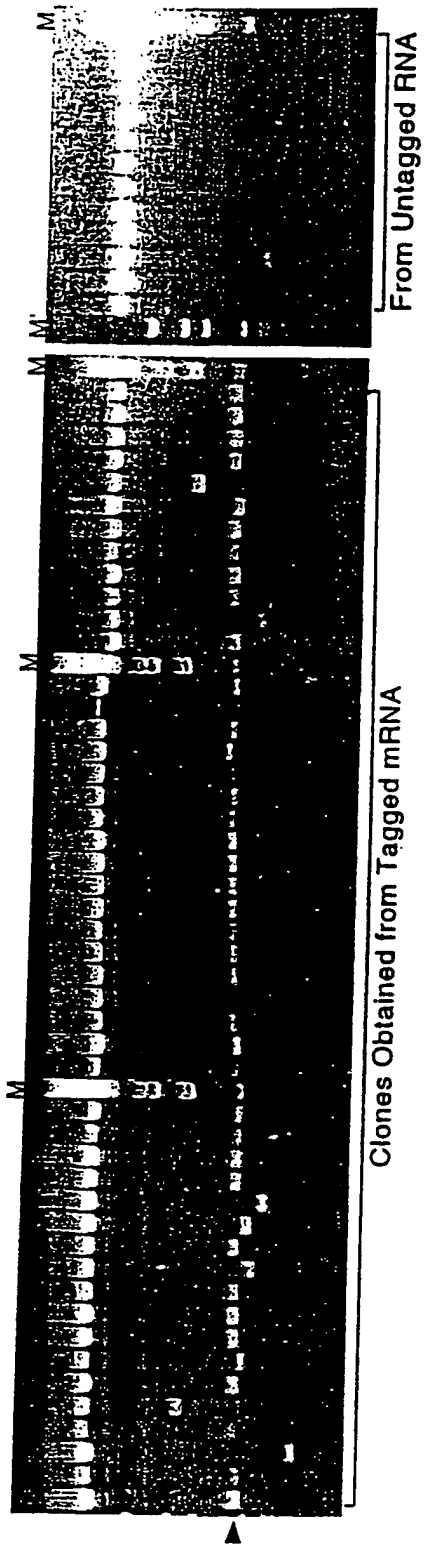

(A)n-OH 3'
RNA Tag Seq.
NVT30
Vector-Primer
Annealing
(A)n-OH 3'
NVT30
Vector-Primer
RNA Tag Seq.
Reverse Transcription
(A)n-OH 3'
NVT30
Vector-Primer
RNA Tag Seq.
RNase I Digestion
Streptavidin Capture
RNase H Digestion
Dilution/Denature/Renature
RNA Tag Seq.
ss cDNA
(A)n
(T)n
ds Vector DNA
RNA Tag Seq.
T4 DNA Polymerase
Gap (A)n
ds cDNA
Gap
(T)n
ds Vector DNA
Transformation into E. coli cells

FIGURE 11

cDNA Synthesis and Cloning: PAVE



Transformation into E. coli cells



>80% Full length inserts for globin mRNA

FIGURE 12

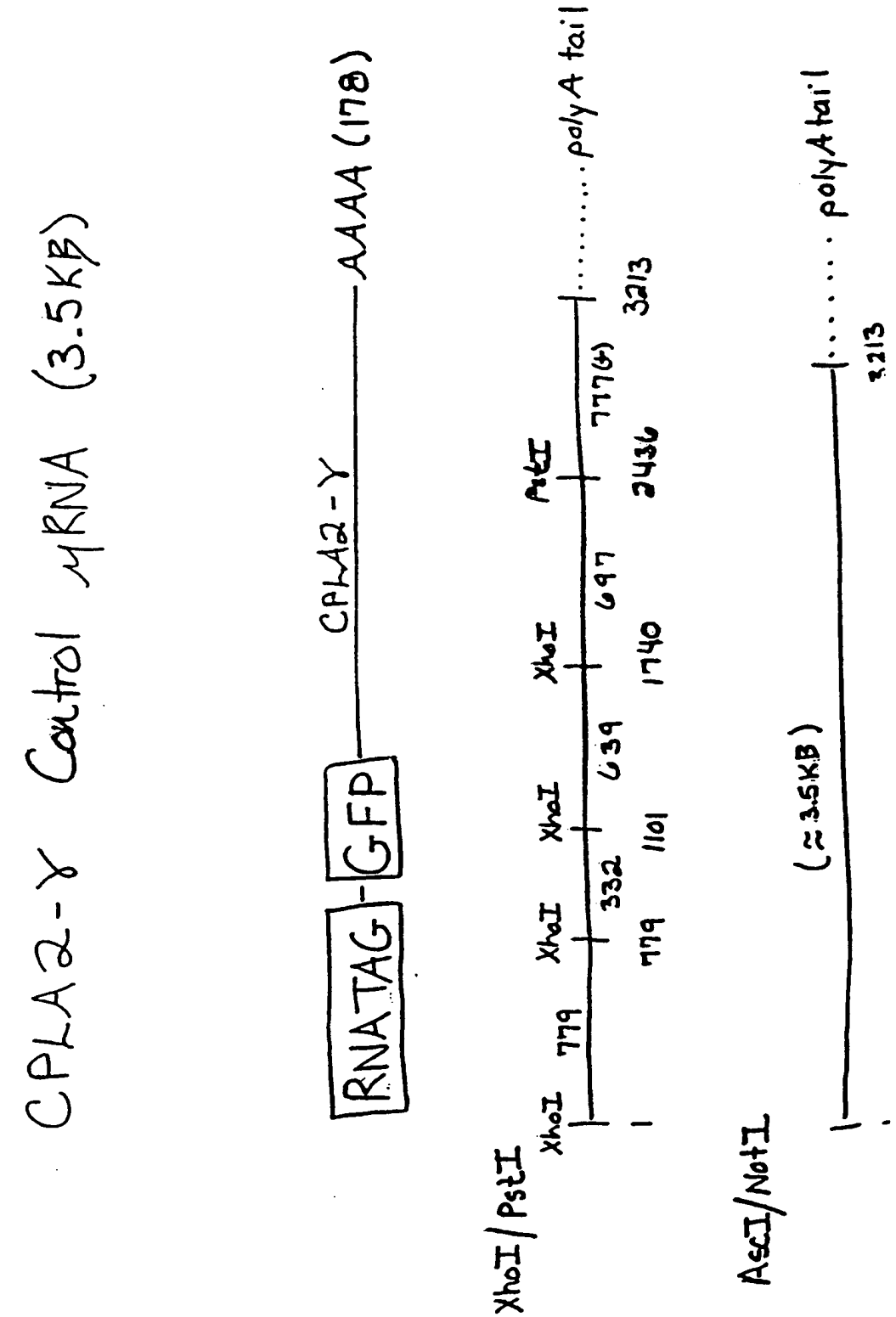

CPLA2-γ Control mRNA (3.5KB)
RNATAG
GFP
CPLA2-γ
AAAA (178)
XhoI/PstI
XhoI
779
1
XhoI
332
779
XhoI
639
1101
XhoI
1740
697
PstI
2436
777(4)
polyA tail
3213
AscI/NotI
(≈3.5KB)
polyA tail
3213

FIGURE 13

FIGURE 14

After RNA:RNA ligation . . . .

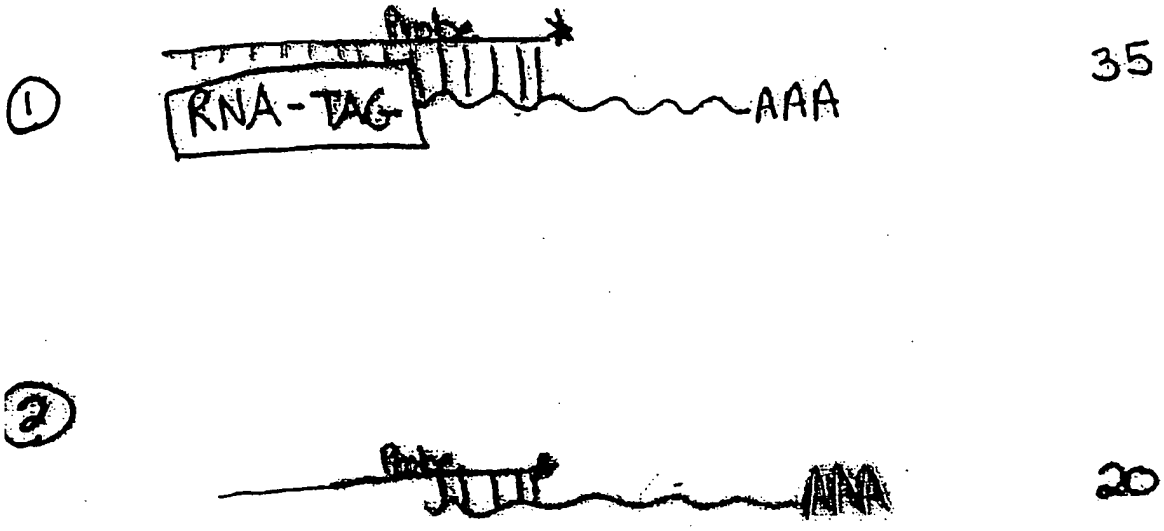
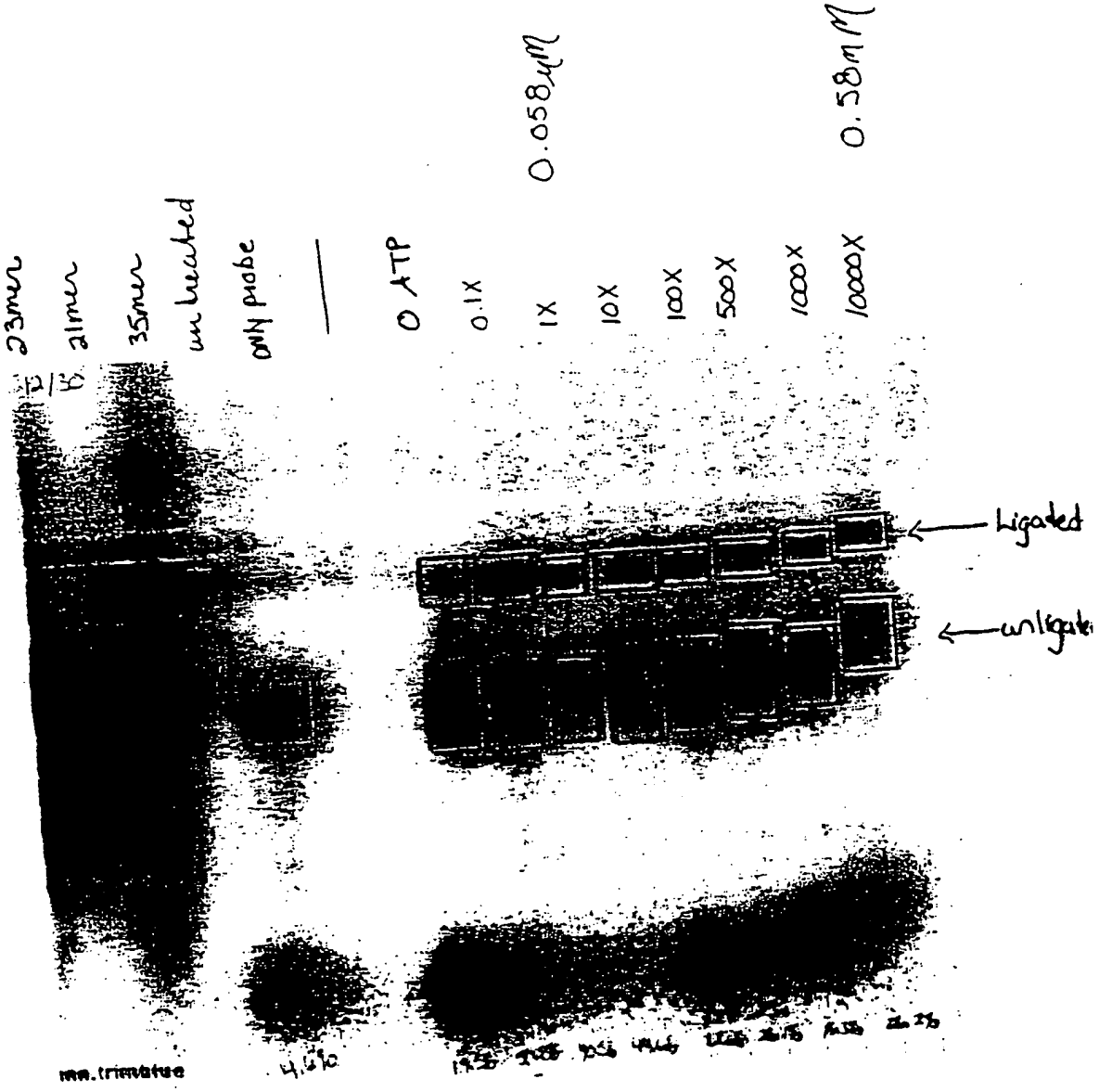
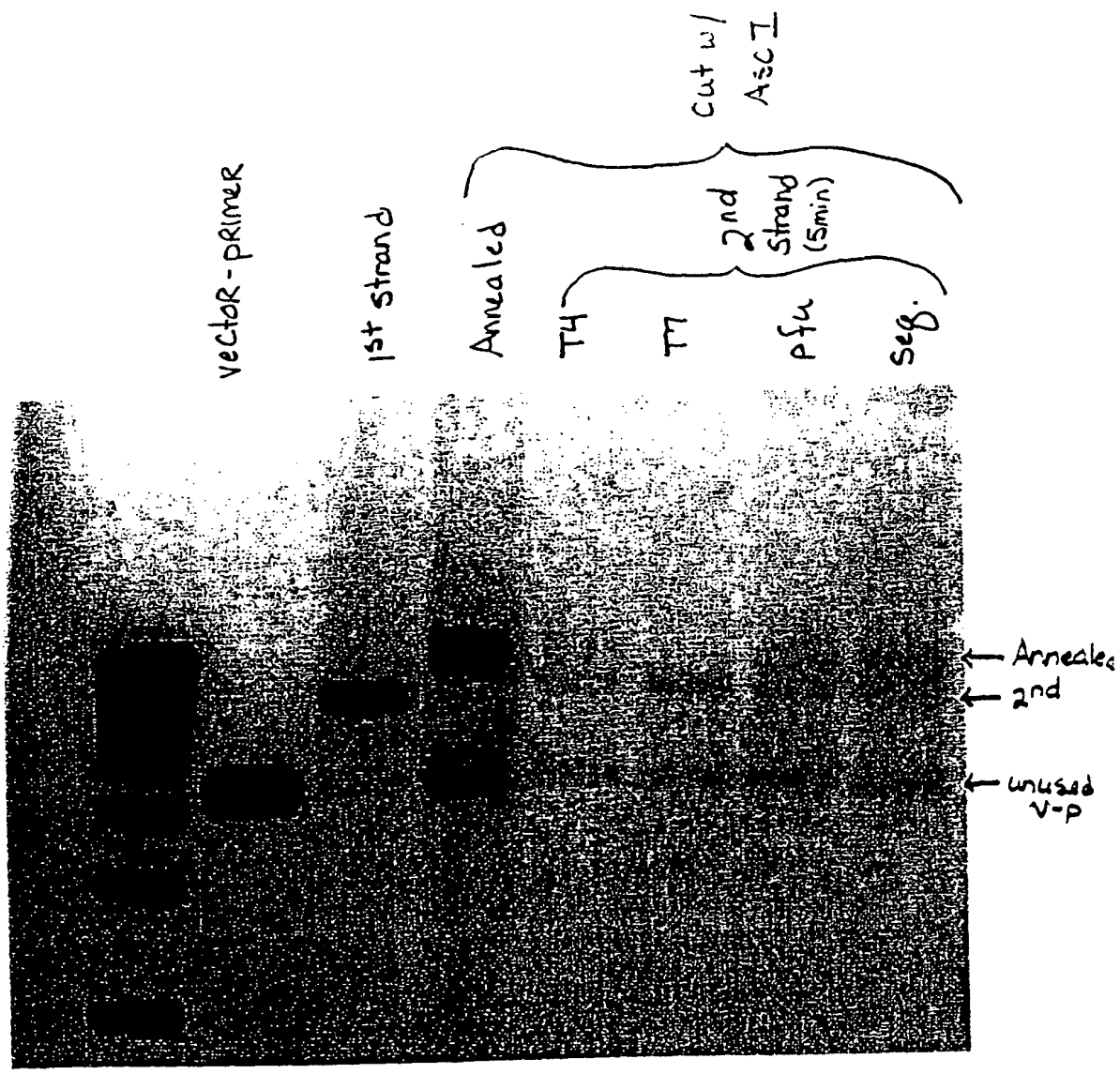


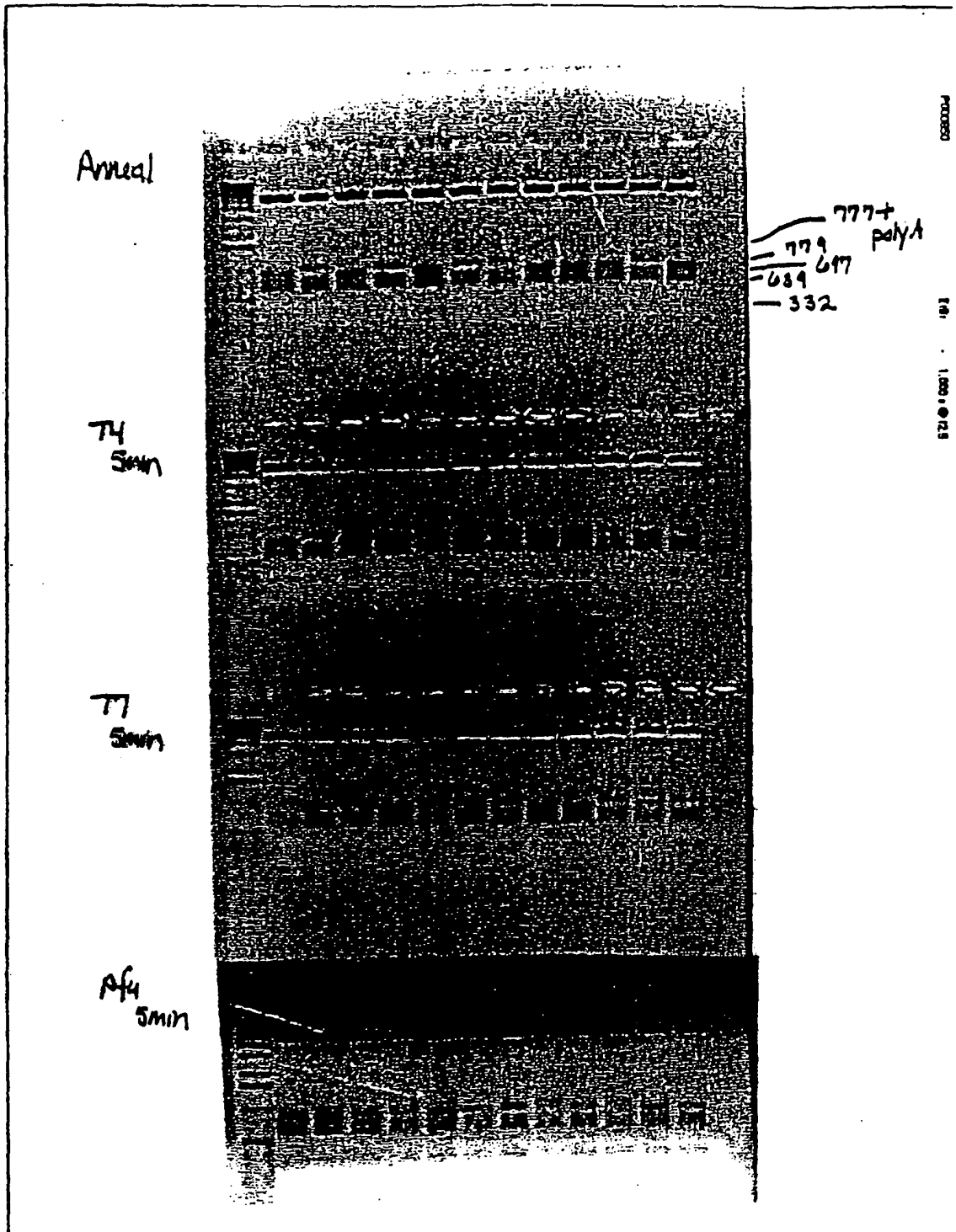
FIGURE 15


23mer
21mer
35mer
unleached
only probe
0 ATP
0.1X
1X
10X
100X
500X
1000X
10000X
0.058μM
0.58mM
Ligated
unligate



* T7 is the ONLY ONE that goes to completion !!

FIGURE 17
* No tRNA inserts!!



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/07332

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12P 19/34; C12M 1/02; C07H 21/02
US CL :435/91.21, 91.51, 320.1; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2, 91.21, 91.51, 183, 320.1; 436/94; 536/23.1, 24.3, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN AND WEST
Search terms: cDNA library, biotin, biotinylation, label, ma, ma-linker, methylguanosine, pyrophosphatase, phosphatase and tag

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KETO et al. Construction of a human full-length cDNA bank. Gene. 1994, Vol. 150, pages 243-250, especially page 244.	1-33
Y	CARNINCI et al. High-efficiency full-length cDNA cloning by biotinylated CAP trapper. Genomics. 1996, Vol. 37, pages 327-336, especially pages 328 and 330.	1-6
Y	STRATAGENE CATALOG, 1994, pages 164, 166, and 170. Published by STRATAGENE CLONING SYSTEMS, 11011 North Torrey Pines Road, La Jolla, CA 92037	23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
18 MAY 2000	06 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FRANK LU Telephone No. (703) 308-1235

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